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Temporal effects of edaravone, a free radical scavenger, on transient ischemia-induced neuronal dysfunction in the rat hippocampus

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Abstract

We examined the effect of a free radical scavenger edaravone on ischemia/reperfusion-induced impairment of long-term potentiation in the perforant path-dentate gyrus synapses of the rat hippocampus, as a measure of functional outcome 4 days after transient global ischemia (2-vessel occlusion, 10 min). Edaravone (3 and 10 mg/kg, i.v.) immediately after reperfusion (Day 0) alleviated ischemia-induced impairment of long-term potentiation in a dose-related manner, whereas treatment on Day 1 or 4 after reperfusion failed to rescue the impaired long-term potentiation. Edaravone administration on Day 0 also prevented the post-ischemic increase in hydroxyl radical formation and the expression of vascular endothelial growth factor, basic fibroblast growth factor and neuronal and inducible nitric oxide synthases of the hippocampus. Thus, edaravone protected the rat hippocampus from ischemia-induced long-term potentiation impairment with a therapeutic time window, suggesting that free radical formation after ischemia/reperfusion is a pivotal trigger of neurofunctional complications after global ischemic stroke.

Keywords: Free radical scavenger; Transient cerebral ischemia; Long-term potentiation; ·OH (hydroxyl radical); NOS (NO synthase); Hippocampus

1. Introduction

Transient global ischemia, arising in humans as a consequence of cardiac arrest or surgery and experimentally induced in animals, leads to delayed brain damage (Kirino, 1982; Pulsinelli et al., 1982; Smith et al., 1984; Yonekura et al., 2004). Transient ischemia has a reperfusion process, which provides oxygen as a substrate for numerous enzyme oxidation reactions that produce free radicals such as super-oxide anion (O_2^-), perhydroxyl radical (HO_2^-), hydroxyl radical (·OH) and free radical nitric oxide (NO). Thus, free radicals are closely related to this type of injury, so-called reperfusion injury (Dirnagl et al., 1999; Nakashima et al., 1999; Lewen et al., 2000; Cuzzocrea et al., 2001; Kontos, 2001).

Cerebral blood flow restoration to the ischemic tissue produces a time-dependent cascade of molecular events including free radical production, however, the kinetics of oxygen-derived free radical formation is extremely complex (Cuzzocrea et al., 2001). Ischemia/reperfusion activates calcium-stimulated enzymes such as proteases and nitric oxide synthases (NOS) (Dirnagl et al., 1999; Lewen et al., 2000). Moreover, ischemia/reperfusion is known to alter vascular function involving the activity of endothelial NOS (eNOS), an enzyme normally crucial in the regulation of vascular and endothelial function (Palmer et al., 1987; Ignarro, 1990; Huang et al., 1994; Lo et al., 1996). Recently, it was also postulated that endothelium-derived NO interacts with free radicals in some circumstances; NO derived from eNOS but not inducible (iNOS) is an essential mediator of delayed preconditioning-induced endothelial protection via its interaction with free radicals (Laude et al., 2003). Thus, a growing body of studies on the pathogenesis of ischemia/

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reperfusion injury has been concerned with alteration of free radical generation or scavenging ability as an essential event for development of ischemic complications, and many treatments have been focused on reducing the formation of these free radicals (Cuzzocrea et al., 2001; Kontos, 2001; Gilgun-Sherki et al., 2002).

Edaravone is a newly developed free radical scavenger for clinical use, which exerts potent free radical quenching activity by trapping a variety of free radical species, especially ·OH (Watanabe et al., 1994; Yamamoto et al., 1997). Namely, edaravone had no effect on O_2^- but apparently trapped ·OH, which is considered to be closely associated with the abilities of this drug as an ·OH scavenger and an antioxidant in ·OH-dependent lipid peroxidation. Indeed, histochemical and biological evidence demonstrated that edaravone prevents post-ischemic brain damage (Abe et al., 1988; Nishi et al., 1989; Oishi et al., 1989; Mizuno et al., 1998).

Numerous studies have evaluated the anti-ischemic properties of agents as indices of the biochemical or morphological alterations including infarction size or neuronal cell death. However, little attention has been focused on the large issue of improving ischemic stroke-induced cerebral dysfunction, as an endpoint of potential efficacy for clinical candidates. We have previously reported that transient global ischemia (2-vessel occlusion, 10 min) produced a delayed synaptic dysfunction, long-term potentiation (LTP) impairment, in the rat hippocampus (Mori et al., 1998; Yoshioka et al., 1999; Togashi et al., 2001). This hippocampal dysfunction was not accompanied by morphological damage in the cerebral regions involving CA1 pyramidal cells (Mori et al., 1998), a structure which is most vulnerable to ischemic insult. Thus, the rat with ischemia and a prolonged reperfusion process is a useful model for evaluating the therapeutic potentials against ischemia/reperfusion-induced cerebral dysfunction.

The aim of the present study was to elucidate the effects of edaravone on ischemia/reperfusion-induced cerebral dysfunction from the viewpoint of a therapeutic time window. Long-term potentiation in perforant path-dentate gyrus synapses in the ischemic rat hippocampus was evaluated as a functional outcome after transient cerebral ischemia. The effects of edaravone were further evaluated by measures of hydroxyl radical formation and protein expressions for three NOS isoforms (eNOS, neuronal NOS (nNOS) and iNOS) and key angiogenic molecules (vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)), in the rat hippocampus after ischemia/reperfusion.

2. Materials and methods

2.1. Animals

Experiments were performed in male Wistar-strain rats (slc:Wistar/ST, Shizuoka Laboratory Animal Center, Hamamatsu, Japan) at an age of 10 to 12 weeks. The animals were housed at an ambient temperature of 22±2 °C under a daily 12-h light–dark cycle with free access to food and water. All animals were handled in compliance with the institutional guidelines, and the experimental procedures were approved by the Animal Research Committee for the Care and Use of Laboratory Animals in the Hokkaido University Graduate School of Medicine.

2.2. Introduction of transient cerebral ischemia

Rats were anesthetized with 1% halothane in a 20% O_2 and 80% N_2 mixture, and were subjected to a 10-min ischemia. Namely, the bilateral common carotid arteries were exposed and clamped with surgical clips. Carotid artery circulation was restored by releasing the clip following a 10-min occlusion. The same surgery was performed in the sham-operated rats but without the carotid artery occlusion. Rectal temperature was monitored throughout surgery by a rectal probe, and maintained at 37 to 38 °C using an electric heating pad. They were then returned to their home cage and allowed free access to food and water.

2.3. Preparation of edaravone

Edaravone (Mitsubishi Pharma, Osaka, Japan) was freshly prepared by dissolving in 1 N NaOH, and adjusting the pH to 7.0 with 1 N HCl and the final concentration of 3 mg/ml or 5 mg/ml with distilled water. Edaravone, at a dose of 3 mg/kg or 10 mg/kg, was administered immediately (Day 0), or 24 h (Day 1) and 96 h (Day 4) after reperfusion via the tail vein.

2.4. Electrophysiological recording

The recording of long-term potentiation was conducted on Day 4 of ischemia/reperfusion (96 h after reperfusion). The rats were anesthetized with 1% halothane, tracheotomized and artificially respired. The animals were fixed in a stereotaxic frame according to bregma and lambda in the same horizontal plane, and blood pressure, heart rate and rectal temperature were monitored throughout the experiment. A stainless steel bipolar stimulating electrode was inserted into the perforant path (8.1 mm posterior, 4.4 mm lateral to the bregma). A recording electrode was inserted into the dentate gyrus (3.5 mm posterior, 2.0 mm lateral to the bregma). Field responses, evoked at a frequency of 0.1 Hz and 250 μs duration, were amplified and monitored with an oscilloscope (VC-10, Nihon Koden, Tokyo, Japan). The responses were averaged with a data analyzing system (MASSCOMP, Concurrent, Tokyo, Japan) and the population spike amplitude was measured. The stimulation intensity for test and train pulses was set to the level that produced a 50–60%

response of the maximum population spike amplitude, as determined before the series of test pulses. Single test stimuli were delivered at intervals of 30 s and the average of five responses was obtained every 5 min. High-frequency (tetanic) stimulation consisted of 5 or 10 trains at 1 Hz, each composed of eight pulses at 400 Hz. After tetanic stimulation, the population spike amplitude was measured for 60 min. Area under the curve was determined from 0 to 60 min after tetanic stimulation as an ensemble effect of treatment.

2.5. Determination of hydroxyl radical formation

As an index of hydroxyl radical formation, 2,3-dihydroxybenzoic acid (DHBA), a non-enzymatic and stable product of salicylic acid, which is formed by scavenging hydroxyl radical (Grootveld and Halliwell, 1986; Halliwell et al., 1991; Ingelman-Sundberg et al., 1991), was quantified in microdialysis samples using a high-performance liquid chromatography (HPLC) with an electrochemical detection (ECD) system (Floyd et al., 1984; Negishi et al., 2001).

Under ketamine anesthesia (100 mg/kg, i.p), a microdialysis guide cannula was stereotaxically implanted into the rat hippocampus. The coordinates relative to the bregma were 5.8 mm posterior, 3.0 mm lateral and 3.3 mm ventral from the dura at 30° from the ventral axis, as described previously (Togashi et al., 1998, 2001). The guide cannula was fixed in the place with a stainless steel anchor screw and dental acrylic cement. The rats were allowed to recover for at least 2 days before experiments. A concentric microdialysis probe was perfused at a constant perfusion flow rate of 2 μg/min, with 5 mM sodium salicylate in Ringer's solution as an $^{\cdot}OH$ trapping agent. Dialysates were collected at 15 min intervals after an equilibration period of 2 h.

After baseline collection for 60 min, rats were subjected to a 10-min ischemia, followed by reperfusion. The rats were freely moving in the microdialysis chamber, except during a 15-min period for the brief ischemia/reperfusion procedure (10 min) performed under halothane anesthesia. The probe placement was confirmed by visual observation after the experiment.

All the samples were analyzed via an on-line HPLC–ECD system (EP-10, ECD-100, EICOM, Kyoto, Japan), which consisted of an HPLC with an electrochemical detector (EP-10 and ECD-100, EICOM, Kyoto, Japan), and a SC-5ODS column (3.0×150 mm, EICOM, Kyoto, Japan). The 3,4-DHBA was detected at potentials of 500 mV. The mobile phase, delivered at 0.5 ml/min, was composed of 98% (v/v) 0.1 M phosphate buffer (pH 6.0), 2% (v/v) HPLC-grade methanol and 50 mg/L EDTA·2 Na (Wako Pure Chemical Industries, Japan).

The concentration of 2,3-DHBA in the dialysate obtained before ischemia was used as a baseline value, and changes were expressed as percentage increase over the baseline value. Maximum response during a 180-min reperfusion period was also evaluated.

2.6. Immunofluorescence staining

Immunohistochemical studies were performed on Day 4 (96 h after reperfusion) with the following commercially available antibodies: anti-human VEGF rabbit polyclonal antibody (Immunological Laboratories, Fujioka, Japan), anti-bovine bFGF mouse monoclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA), anti-human eNOS rabbit polyclonal antibody (Affinity BioReagents, Golden, CT, USA), anti-human nNOS rabbit polyclonal antibody (ZYMED Laboratories, San Francisco, CA, USA) and anti-rabbit iNOS mouse monoclonal antibody (Affinity BioReagents, Golden, CT, USA).

For immunofluoroscence staining of the target molecules, hippocampal tissues were rapidly removed from sham-operated control rats and vehicle-treated or edaravone (10 mg/kg, i.v.)-treated (Day 0) ischemic rats under ketamine (100 mg/kg, i.p.) anesthesia, and frozen in liquid nitrogen. The preparations were cut in 10 μm thick sections transversely. The frozen cryostat sections were then fixed in acetone for 10 min at 4 °C and air-dried. To prevent non-specific staining by the secondary antibody, the sections were blocked by non-immune serum (1% bovine serum albumin in Tris) for 30 min at room temperature. After overnight incubation at 4 °C with primary antibodies, the sections were rinsed in phosphate buffer solution and then exposed to the fluorescence secondary antibody, Cy3-conjugated AffiniPure anti-rabbit IgG or fluorescein-conjugated AffiniPure anti-mouse IgG (Jackson Immuno Research Laboratories, Westgrove, PA, USA), for 2 h according to the manufacturer's instructions. The samples processed without primary antibodies served as negative controls. The coverslips were mounted with Immunon (Thermo Shandon, Pittsburgh, PA, USA). Immunofluorescence images were observed under the laser scanning confocal imaging system (MRC-1024; Bio-Rad, Hemel Hempstead, UK).

2.7. Western blot analysis

Immunoblot analysis of protein expressions for angiogenic molecules and NOS isoforms was performed on Day 4 (96 h after reperfusion) with antibodies used for immunofluorescence staining. Hippocampal tissues that had been removed from sham-operated control rats and vehicle-treated or edaravone (10 mg/kg, i.v.)-treated (Day 0) ischemic rats under ketamine (100 mg/kg, i.p.) anesthesia and rinsed in sterilized water on ice, were minced with scissors, homogenized and then centrifuged at 500 $\times g$ for 15 min to pellet any insoluble material. The protein concentration of supernatant was determined with the bicinchoninic acid protein assay (Pierce, Ill., USA). Samples were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), using 7.5–15% polyacrylamide gel, and electrotransferred to polyvinylidine difluoride filter (PVDF) membrane. To

reduce nonspecific binding, the PVDF was blocked for 2 h at room temperature with 5% non-fat milk in buffer solution (phosphate-buffered saline containing 0.1% Tween 20). Thereafter, the PVDF was incubated overnight at 4 °C with specific antibodies for VEGF, bFGF, eNOS, nNOS and iNOS (1:100–1000 dilution) in buffer solution. After washing three times with buffer solution, the PVDF was incubated with horseradish peroxidase-conjugated anti-rabbit (Amersham) or anti-mouse (Amersham) antibody diluted at 1:2000–10,000 in buffer solution at room temperature for 60 min. Then, the PVDF was washed five times in buffer solution. The blots were developed using an enhanced chemiluminescence detection system (Amersham). The chemiluminescence was visualized using a Lumino Image Analyzer (LAS1000, Fuji Photo Film, Tokyo, Japan) or exposed to X-ray film (Fuji Photo Film). To check for protein loading/transfer variations, all blots were stained with Ponceau Red (washable, before incubation with antibodies) and with Coomassie brilliant blue (permanent, after the enhanced chemiluminescence detection system). The intensity of total protein bands per lane was evaluated by densitometry. Negligible loading/transfer variation was observed between samples.

2.8. Statistical analysis

All values are expressed as mean±S.E.M. The statistical significance of differences between group means was determined by a one-way or repeated measure analysis of variance (ANOVA). The significance of differences among all groups was assessed with the Tukey's post-hoc multiple comparison test. Differences were considered to be significant when $P<0.05$.

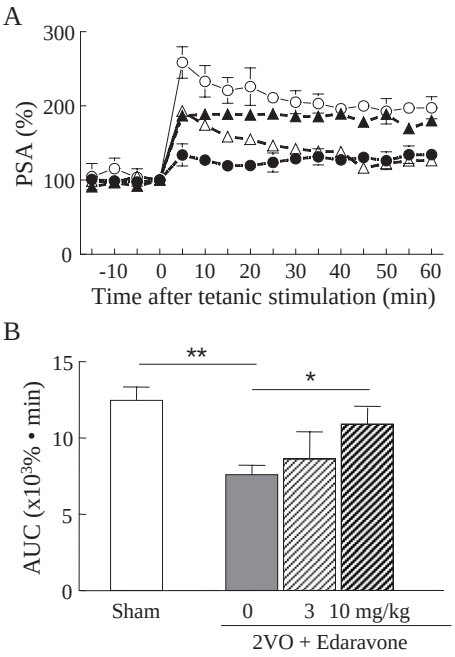

Fig. 1. Dose-dependent effects of edaravone on ischemia/reperfusion-induced long-term potentiation in perforant path-dentate gyrus synapses of the rat hippocampus. (A) Time course changes and (B) the area under the curves of the population spike amplitude (PSA) during the 60-min (AUC_{60min}) after tetanic stimulation. Edaravone (3 or 10 mg/kg) or vehicle was injected into the tail vein of the rat with 2-vessel occlusion (2VO) or sham-operation (Sham) immediately after reperfusion (Day 0). Long-term potentiation was recorded on Day 4 (96 h after reperfusion). Open circles: Sham, filled circles: vehicle-treated 2VO, open triangles: edaravone (3 mg/kg, i.v.)-treated 2VO, filled triangles: edaravone (10 mg/kg, i.v.)-treated 2VO. Data are expressed as the mean±S.E.M. of a percentage of the PSA obtained before tetanic stimulation of 5 to 8 rats. *$P<0.05$, **$P<0.01$ (in the panel A showing the time course changes, some error bars and marks indicating statistical significance were not presented to reduce the complexity of the figure).

3. Results

3.1. Long-term potentiation formation

Hippocampal long-term potentiation in the perforant path-dentate gyrus synapses determined 4 days after reperfusion was markedly impaired in ischemic rats, as compared with sham-operated control rats (Fig. 1A,B). Edaravone (3 and 10 mg/kg, i.v.), injected immediately after reperfusion (Day 0), improved long-term potentiation induction in a dose-dependent manner. Area under the curve for 60 min in the high dose (10 mg/kg) edaravone-treated ischemic rat ($12.46\pm0.88\times10^3\%\cdot$min, $n=6$, $P<0.05$), was significantly different from that in the vehicle-treated ischemic rat ($7.59\pm0.62\times10^3\%\cdot$min, $n=8$), and was identical to that in the sham-operated rat ($10.89\pm1.06\times10^3\%\cdot$min, $n=7$) (Fig. 1B).

Ameliorating effects of edaravone on ischemia/reperfusion-induced long-term potentiation impairment were evaluated from the viewpoint of a therapeutic time window. Edaravone, at a dose of 10 mg/kg, was administered immediately (Day 0), 24 h (Day 1) or 96 h (Day 4) after reperfusion. Significant effects of edaravone obtained by the Day 0 treatment, were not observed in the ischemic rat treated on Day 1 and Day 4 (Fig. 2A,B). Analysis of area under the curve for 60 min after tetanic stimulation showed that long-term potentiation was slightly improved in the Day 1-treated group, however, it was not significant compared to the vehicle-treated ischemic rat (Fig. 2B).

3.2. Study on hydroxyl radical formation

The hippocampal level of hydroxylation products of salicylic acid, 2,3-DHBA as a measure of hydroxyl radical formation, slightly increased after ischemia/reperfusion in the vehicle-treated ischemic rat, whereas it gradually decreased in the vehicle-treated sham-operated rat. The peak production of hydroxyl radical in the ischemic rat hippocampus was observed during the first 60-min period of

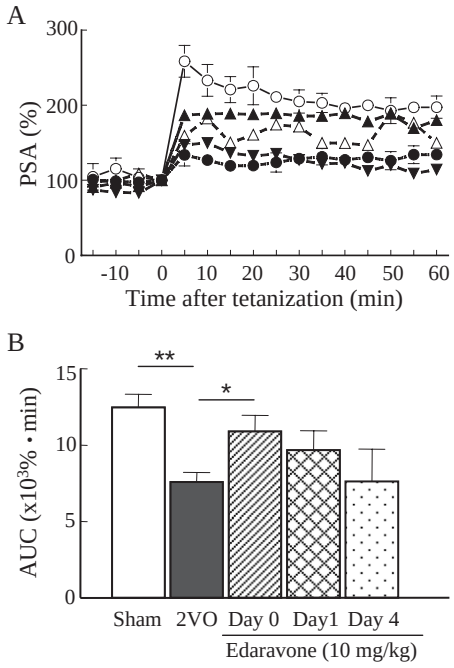

Fig. 2. Temporal effects of edaravone on ischemia/reperfusion-induced long-term potentiation in perforant path-dentate gyrus synapses of the rat hippocampus. (A) Time course changes and (B) the area under the curves of the population spike amplitude (PSA) during the 60-min (AUC_{60min}) after tetanic stimulation. Edaravone (10 mg/kg) or vehicle was injected into the tail vein of the rat with 2-vessel occlusion (2VO) or sham-operation (Sham) immediately (Day 0), 24 h (Day 1) and 96 h (Day 4) after reperfusion. Long-term potentiation was recorded on Day 4. Open circles: Sham, filled circles: vehicle-treated 2VO, filled triangles: edaravone (Day 0)-treated 2VO, open triangles: edaravone (Day 1)-treated 2VO, inverse filled triangles: edaravone (Day 4)-treated 2VO. Data are expressed as the mean±S.E.M. of a percentage of the PSA obtained before titanic stimulation (tetanus) of 5 to 8 rats. *P<0.05, **P<0.01 (in the panel A showing the time course changes, some error bars and marks indicating statistical significance were not presented to reduce the complexity of the figure).

reperfusion. As shown in Fig. 3, the maximum response of 2,3-DHBA (116.1±5.0%, n=8) was significantly higher than that in the vehicle-treated sham-operated rat (99.5±0.6%, n=10; P<0.01). In the edaravone (10 mg/kg, i.v.)-treated ischemic rat, the time course changes and the maximum response of the hippocampal 2,3-DHBA levels were comparable to those in the vehicle-treated sham-operated group. The maximum response of 2,3-DHBA (98.0±2.0%, n=6) was significantly (P<0.01) different from that in the vehicle-treated ischemic rats (Fig. 3B). Edaravone treatment immediately after reperfusion (Day 0), thus, counteracted the 10-min ischemia-induced hydroxyl radical formation.

3.3. Immunoblot analysis on expression of NOS and angiogenic molecules

Immunoblot analysis using the antiserum raised against human or rabbit NOS (eNOS, nNOS and iNOS) showed single bands with molecular masses of approximately 130 kDa, 160 kDa, 140 kDa, 39 kDa and 180 kDa, which were referred to as eNOS, nNOS, iNOS, VEGF and bFGF, respectively (Fig. 4). The bands obtained from the vehicle-treated ischemic rat hippocampus on Day 4 were more pronounced than those from the sham-operated rat hippocampus. Densitometric quantification of the signals revealed that NOS and angiogenic molecule protein levels in the vehicle-treated ischemic rat were 259.7±10.4% for eNOS, 258.1±19.4% for nNOS, 257.0±5.0% for iNOS, 173.8±5.3% for VEGF and 154.9±6.6% for bFGF (n=10, P<0.01 vs. the sham-operated rat). The increased expression levels of NOS and angiogenic molecules except eNOS were significantly reduced when ischemic rats were treated with edaravone (10 mg/kg) on Day 0. Densitometric analysis showed that nNOS, iNOS and angiogenic molecule protein levels in the edaravone-treated (Day 0) ischemic rat were 177.0±11.8% for nNOS, 132.1±7.1% for iNOS, 97.0±8.0% for VEGF and 90.92±4.3% for bFGF (n=10, P<0.01 vs. the vehicle-treated ischemic rat). Conversely, edaravone treatment on Day 0 resulted in a marked and significant increase in eNOS expression of the ischemic rat hippocampus (571.3±36.4%, n=5, P<0.001 vs. the sham-operated rat and the vehicle-treated ischemic rat) (Fig. 4C).

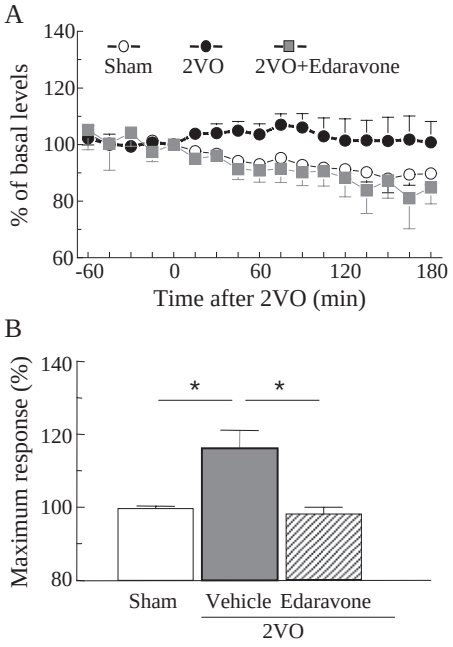

Fig. 3. Effects of edaravone on hydroxyl radical formation in the rat hippocampus after ischemia/reperfusion. The hippocampal level of hydroxylation product of salicylic acid, 2,3-dihydroxybenzoic acid (DHBA), as a measure of hydroxyl radical formation before and after ischemia/reperfusion. (A) Time course changes and (B) the maximum response after reperfusion in 2,3-DHBA levels. Edaravone (10 mg/kg) or vehicle was injected into the tail vein of the rat with 2-vessel occlusion (2VO) or sham-operation (Sham) immediately (Day 0) after reperfusion. Data are expressed as the mean±S.E.M. of a percentage of the value obtained before ischemia/reperfusion of 6 to 8 rats. *P<0.05 (in the panel A showing the time course changes, some marks indicating statistical significance were not presented to reduce the complexity of the figure).

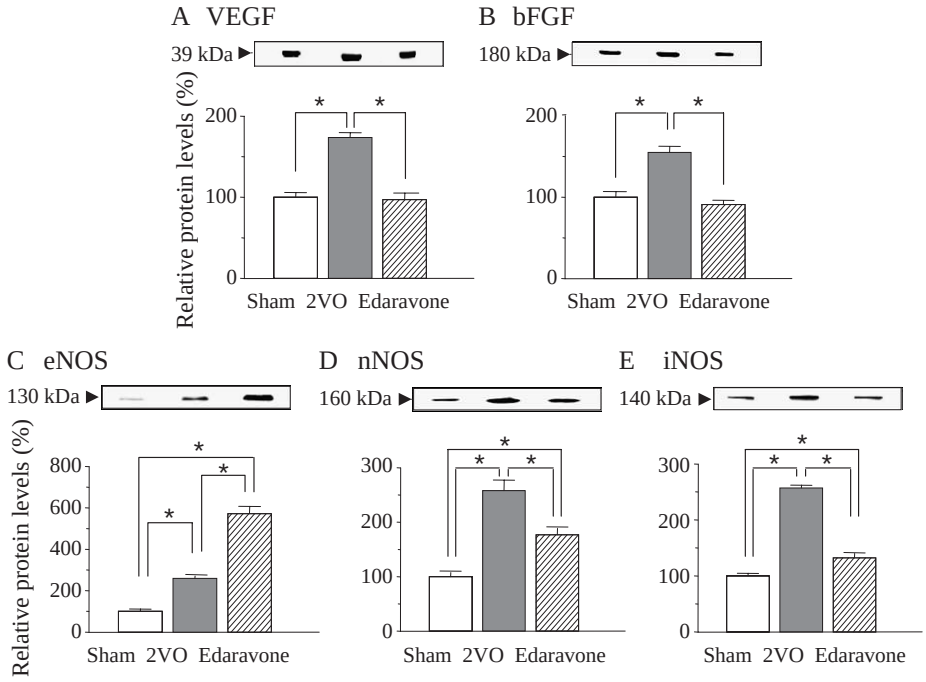

Fig. 4. Immunoblot analysis for angiogenic growth factors and nitric oxide synthases (NOS) in the rat hippocampus after ischemia/reperfusion. Angiogenic molecules, (A) vascular endothelial growth factor (VEGF) and (B) basic fibroblast growth factor (bFGF), and NOS isoforms, (C) endothelial NOS (eNOS), (D) neuronal NOS (nNOS) and (E) inducible NOS (iNOS), were evaluated by immunoblot analysis on Day 4 (96 h after reperfusion). Edaravone (10 mg/kg) was injected into the tail vein of the rat with 2-vessel occlusion (2VO) immediately after reperfusion (Day 0). The upper trace of each panel shows representative blots of the respective protein obtained from the sham-operated control rat (Sham) (lane-1), and the vehicle-treated 2VO rat (2VO) (lane-2) and the edaravone (10 mg/kg, i.v.)-treated 2VO rat (Edaravone) (lane-3). The experiments were conducted by loading equal amounts of the respective protein. The lower trace of each panel shows the bar graph summarizing the immunoblot data. Densitometric results are expressed as a percentage of each band obtained from the sham-operated rat. Data are shown as the mean±S.E.M. of five experiments. *P<0.01.

3.4. Immunofluorescent analysis on expression of NOS and angiogenic molecules

The results of immunofluorescent studies were coincided well with those of immunoblotting as described above. Immunofluorescent analysis showed that abundant NOS and angiogenic molecule protein expressions were obvious in the vehicle-treated ischemic rat hippocampus compared to those in the sham-operated rat. In the ischemic rat treated with edaravone (10 mg/kg) on Day 0, hippocampal expressions of nNOS, iNOS, VEGF and bFGF were normalized to those in the sham-operated rat. On the contrary, eNOS protein expression was not down-regulated but rather up-regulated in the hippocampus of the edaravone-treated rat (Fig. 5).

4. Discussion

We demonstrated here that a free radical scavenger edaravone alleviated the ischemia/reperfusion-induced cerebral dysfunction, by evaluating long-term potentiation in the dentate gyrus synapses as a functional neurological outcome of the rat hippocampus. The effects of edaravone were time-dependent; treatment in the early (just after reperfusion; Day 0), but not late (24 or 96 h after; Day 1 or Day 4) stage of reperfusion, significantly improved long-term potentiation. Day 0 treatment with edaravone altered NOS features observed in the vehicle-treated ischemic rat, which showed up-regulation of eNOS, nNOS and iNOS protein expressions. Edaravone down-regulated nNOS and iNOS expressions, whereas it further up-regulated eNOS expression. Taking a detrimental/beneficial role of NOS isoforms in ischemic brain injury into consideration, the present findings suggest that edaravone protects the rat hippocampus from ischemia/reperfusion-induced neurofunctional damage with a therapeutic time window.

Ischemia/reperfusion is known to produce reactive oxygen and nitrogen species including hydroxyl radical, superoxide anion and free radical NO, which can cause widespread damage to cellular components such as lipids, proteins and DNA, leading to subsequent necrosis or apoptosis (Dirnagl et al., 1999; Nakashima et al., 1999; Lewen et al., 2000; Cuzzocrea et al., 2001; Kontos, 2001). However, the kinetics of oxygen-derived free radical formation is extremely complex (Cuzzocrea et al., 2001), and currently available evidence is limited, especially regarding the relationship between temporal profiles of free radical generation and cerebral dysfunction after ischemic stroke. Edaravone, a free radical scavenger, has been proven to show prominent antioxidant and free radical scavenging properties (Watanabe et al., 1994). Histochemical and

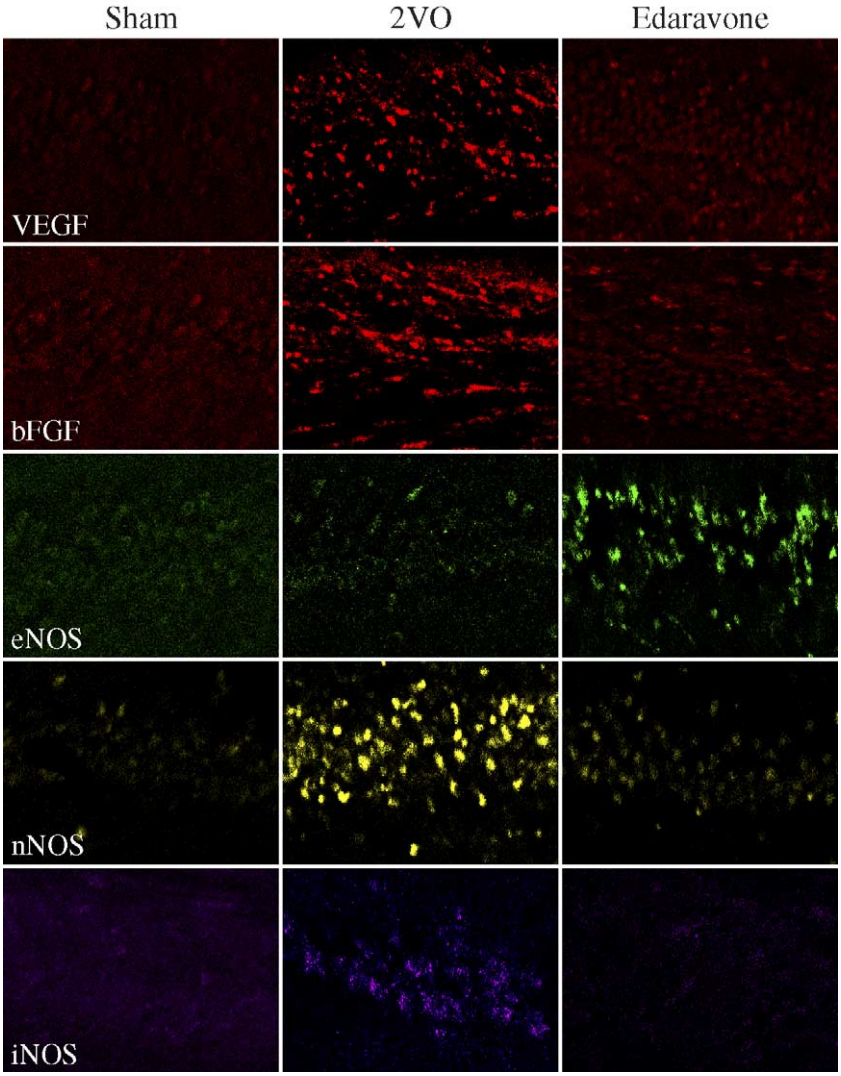

Fig. 5. Confocal images showing immunofluorescence staining for angiogenic growth factors and nitric oxide synthase (NOS) isoforms in the rat hippocampus after ischemia/reperfusion. Angiogenic molecules, (A) vascular endothelial growth factor (VEGF) and (B) basic fibroblast growth factor (bFGF), and NOS isoforms, (C) endothelial NOS (eNOS), (D) neuronal NOS (nNOS) and (E) inducible NOS (iNOS), were evaluated by immunofluorescence staining on Day 4 (96 h after reperfusion). Edaravone (10 mg/kg) or vehicle was injected into the tail vein of the rat with 2-vessel occlusion (2VO) or sham-operation (Sham) immediately after reperfusion (Day 0).

biological evidence demonstrated that edaravone prevented postischemic edema and disrupted monoamine metabolism (Abe et al., 1988; Nishi et al., 1989; Oishi et al., 1989; Mizuno et al., 1998). In patients with cerebral infarction, edaravone also prevented cerebral damage possibly by free radical scavenging (Houkin et al., 1998). We show here that edaravone treatment in the early (Day 0) but not late stage of reperfusion ameliorated the hippocampal long-term potentiation in the rat with 10-min cerebral ischemia, an incomplete global ischemic model without pathological changes in the hippocampal regions (Mori et al., 1998). This indicated that edaravone protected the hippocampus from ischemia/reperfusion-induced neuronal dysfunction with a therapeutic time window. Edaravone is known to trap a variety of free radical species, particularly, hydroxyl radical, which is considered to be closely associated with the abilities of this drug as an $^{\cdot}OH$ scavenger and an antioxidant in $^{\cdot}OH$-dependent lipid peroxidation (Watanabe et al., 1994; Yamamoto et al., 1997). Indeed, we demonstrated in the present study that hydroxyl radical formation, estimated from 2,3-DHBA levels observed in the vehicle-treated ischemic rat, was significantly counteracted by edaravone treated immediately after reperfusion (on Day 0). Thus, our findings provide a direct evidence to strongly suggest that hydroxyl radical is a critical molecule as a trigger of the process in neurofunctional damage after ischemic stroke. However, we should consider the possibility that free radical scavenging activities might not fully explain the protective effect of edaravone against ischemic neuronal dysfunction, as pointed out against ischemic brain injury (Wu et al., 2000).

O_2 is an important determinant of NOS activity in the hypoperfused tissue. Numerous reports have concerned

differential roles of NOS isoforms or their temporal NO production in the pathogenesis for ischemic brain injury (Zhang et al., 1995; Iadecola, 1997; Santizo et al., 2000; Jiang et al., 2002). eNOS-derived NO is thought to be beneficial to promote collateral circulation and microvascular flow, whereas nNOS- and iNOS-derived NO is detrimental in the ischemic brain. We have previously reported that cerebral functional damage after transient global ischemia was at least in part due to nitrosative stress via up-regulated iNOS, since preventing post-ischemic NO production by an iNOS inhibitor aminoguanidine resulted in restoring long-term potentiation in the post-ischemic hippocampus (Mori et al., 1998; Yoshioka et al., 1999; Togashi et al., 1998, 2001). In the present study, transient ischemia up-regulated expression of all three NOS isoforms at protein levels when evaluated 4 days after ischemic stroke. Edaravone treatment on Day 0 down-regulated nNOS and iNOS expressions, while it up-regulated eNOS expression observed in the vehicle-treated ischemic rat. Assessing the beneficial/detrimental roles of the three NOS-derived NO, edaravone is likely to alter the post-ischemic NOS features to a preferable one. In other words, the alleviating effect of edaravone on ischemia-induced long-term potentiation impairment might be partly supported by our findings on hippocampal NOS features of up-regulated eNOS and down-regulated nNOS and iNOS expressions.

It is of note that edaravone did not counteract the increased eNOS expression in the ischemic rat, rather further up-regulated. Although we cannot explain the mechanism mediating eNOS expression following edaravone treatment in the ischemic rat, it is likely to be supported by the recent reports that edaravone exerted eNOS induction and prevented spinal cord damage after transient ischemia (Takahashi et al., 2003), and normalized irradiation-reduced eNOS expression as well as endothelium-dependent relaxation in the rabbit central artery (Zhang et al., 2003). eNOS is an enzyme crucial in the regulation of vascular and endothelial function (Fleming and Busse, 1999). Potential mechanisms for eNOS are thought to be augmentation of blood flow, e.g., vasodilation of cerebral vessels supplying the ischemic tissue, leukocyte–endothelial interactions or platelet–endothelial interactions (Atochin et al., 2003). Recently, it has been postulated that endothelium-derived NO interacts with free radicals in some circumstances; NO derived from eNOS but not iNOS is an essential mediator of delayed preconditioning-induced endothelial protection via its interaction with free radicals (Laude et al., 2003). Edaravone-induced eNOS up-regulation as well as nNOS/iNOS normalization in the ischemic rat, therefore, might be contributing to its alleviating effects on long-term potentiation, although the molecular mechanism whereby it ameliorates the ischemic dysfunction remains to be identified.

It is known that some angiogenic molecules, including VEGF and bFGF and their receptors, increase after cerebral ischemia (Kovacs et al., 1996; Marti et al., 2000; Leker et al., 2001; Wei et al., 2001; Hayashi et al., 2003). The present study demonstrated that transient ischemia up-regulated expression of angiogenic molecules, VEGF and bFGF, at protein levels 4 days after ischemic stroke. Edaravone treatment on Day 0 down-regulated both angiogenic molecules. Although the pathophysiological significance for the effects of edaravone on the ischemia-induced features of these angiogenic molecules is still unknown, it might indicate that edaravone prevented the progression of ischemia-induced cerebrovascular remodeling in the post-ischemic hippocampus. It is noteworthy that edaravone produced a dissociative effect between eNOS and VEGF expression, though the anatomical and temporal similarity of VEGF and eNOS induction has been reported in the brain after permanent ischemia (Leker et al., 2001). It might be postulated that edaravone up-regulated eNOS expression via a mechanism(s) separate from that for angiogenic molecule expression. Further study is needed to shed light on the mechanism(s) involved in edaravone-induced eNOS induction and the relationship between the changes in the hippocampal features of angiogenic molecule expression and the ameliorating effects on ischemia-induced impairment of long-term potentiation by edaravone.

In conclusion, we demonstrated that edaravone, a free radical scavenger, prevented the impaired long-term potentiation in the perfront path-dentate gyrus synapses of the ischemic rat hippocampus, with a therapeutic time window. The alleviating effect of edaravone was also supported by the hippocampal feature of the expression of NOS isoforms and angiogenic molecules and hydroxyl radical formation. We thus assume that oxygen free radicals involving NO and hydroxyl radical are closely related to the ischemia/reperfusion-induced neurofunctional disability observed in the hippocampus of this global ischemic model. Our present findings using edaravone also provide information on the temporal aspects of molecular events underlying cerebral dysfunction after transient global ischemia.

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