Edaravone Reduces Early Accumulation of Oxidative Products and Sequential Inflammatory Responses After Transient Focal Ischemia in Mice Brain

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Background and Purpose—Oxidative stress contributes to ischemia/reperfusion neuronal damage in a consecutive 2-phase pattern: an immediate direct cytotoxic effect and subsequent redox-mediated inflammatory insult. The present study was designed to assess the neuroprotective mechanisms of edaravone, a novel free radical scavenger, through antioxidative and anti-inflammatory pathways, from the early period to up to 7 days after ischemia/reperfusion in mice.

Methods—Mice were subjected to 60-minute ischemia followed by reperfusion. They were divided into the edaravone group (n=72; with different schedules for first administration) and the vehicle (control) group (n=36). Infarct volume and neurological deficit scores were evaluated at several time points after ischemia. Immunohistochemical analysis for 4-hydroxy-2-nonenal (HNE), 8-hydroxy-deoxyguanosine (8-OHdG), ionized calcium-binding adapter molecule 1 (Iba-1), inducible NO synthase (iNOS), and nitrotyrosine were performed at 24 hours, 72 hours, or 7 days after reperfusion.

Result—Edaravone, even when administered 6 hours after onset of ischemia/reperfusion, significantly reduced the infarct volume (68.10±6.24%; \( P<0.05 \)) and improved the neurological deficit scores (\( P<0.05 \)) at 24 hours after reperfusion. Edaravone markedly suppressed the accumulation of HNE-modified protein and 8-OHdG at the penumbra area during the early period after reperfusion (\( P<0.05 \)) and reduced microglial activation, iNOS expression, and nitrotyrosine formation at the late period.

Conclusion—Our results indicated that edaravone exerts an early neuroprotective effect through the early free radicals scavenging pathway and a late anti-inflammatory effect and suggested that edaravone is important for expansion of the therapeutic time window in stroke patients. (Stroke. 2005;36:2220-2225.)

Key Words: ischemia ■ lipid peroxidation ■ microglia ■ nitric oxide synthase ■ reperfusion injury
Materials and Methods

Experimental Protocol

All animal procedures were conducted after gaining the approval of the Animal Care Committee of Juntendo University. Adult 8-week-old male C57BL/6 mice weighing 20 to 22 g were obtained from the Charles River Institute (Kanagawa, Japan) and maintained on a 12-hour light/dark cycle with continuous access to food and water. Mice were divided at random into 4 groups. Mice of the edaravone group (n=36) were treated with tail vein infusion of edaravone (Mitsubishi Wellphama) at 3 mg/kg body weight twice: immediately and 30 minutes after reperfusion of the middle cerebral artery (MCA) occlusion. This treatment schedule and dosage were based on the pharmacokinetic profile of edaravone supplied by the manufacturer and our own preliminary experiments. Mice of the control saline group (n=36) received intravenous infusion of saline solution at a volume similar to that used in the edaravone group. Mice of the control sham-operated vehicle- and edaravone-treated groups (n=8 for each subgroup) underwent the same aforementioned protocol except for MCA occlusion (MCAO). Mice of the therapeutic assessment group (n=12 for each subgroup) underwent the same procedure as above, but the first administration of edaravone was performed on 1-mm-thick coronal brain sections throughout the time window. Ischemia was induced by the intraluminal vascular occlusion method as described previously. Briefly, the left MCA was occluded in anesthetized mice, followed by release of the occlusion 60 minutes later. Regional cerebral blood flow (rCBF) was measured by laser Doppler flowmetry before, during, and after MCAO, as well as before death. Neurological function was assessed using a standard scoring system: 0, no defect; 1, failure to extend right forepaw; 2, circling to right; 3, falling to right; and 4, inability to walk spontaneously. At 24 hours, 72 hours, or 7 days (n=5 each) after reperfusion, 2,3,5-triphenyl tetrazolium chloride (TTC) staining was performed on 1-mm-thick coronal brain sections throughout the brain to evaluate the infarct volume as described previously.

Immunohistochemistry

Immunohistochemistry was performed on 20 μm-thick free-floating coronal sections, which were prepared as described previously. After incubation in 3% H2O2 followed by 10% block ace in 0.1 PBS, the sections were stained overnight at 4°C using a rabbit polyclonal antibody against 4-hydroxy-2-nonenal (HNE; 40:1; Alpha Diagnostic International) to assess lipid peroxidation; a mouse monoclonal antibody against 8-hydroxy-deoxyguanosine (8-OHdG; 100:1; Japan Institute For the Control of Aging) to detect oxidative DNA damage; a rabbit polyclonal antibody against ionized calcium-binding adapter molecule 1 (Iba-1; 500:1; Wako Pure Chemicals) as a marker of active microglia; and a mouse monoclonal antibody against iNOS (250:1; BD Transduction Laboratories). Sections were then treated with secondary antibodies (Vectastain; Vector Laboratories). Immunoreactivity was visualized subsequently by the avidin-biotin complex method (Vectastain; Vector Laboratories) as described previously.

Double Immunofluorescence Histochemistry

Double immunofluorescence staining for nitrotyrosine and neuronal, microglial, or astrocytal marker was performed on selected coronal sections. The primary antibodies were rabbit polyclonal antibody against 3-nitrotyrosine (1:100; Upstate Biotechnology); mouse monoclonal antibody against microtubule-associated protein-2 (1:100; Chemicon International); rat monoclonal antibody against mouse CD11b (Mac-1; 1:100; BD Bioscience); and mouse monoclonal antibody against glial fibrillary acidic protein (GFAP; 1:1000; Sigma Chemical Co.). Alexa Fluor 594–conjugated goat anti-rabbit IgG (1:400; Vector Laboratories) was used to demonstrate neuron, microglia, and astrocyte immunoreactivity as green fluorescence. Iba-1,–conjugated horse anti-mouse and anti-rat IgG (1:400; Molecular Probes) to demonstrate neuron, microglia, and astrocyte immunoreactivity as red fluorescence.

SDS-PAGE and Immunoblotting

Proteins were extracted from the penumbra area and measured as described previously. Aliquots containing 30 μg of protein were subjected to 10% SDS-PAGE. The protein bands were transferred to polyvinylidene difluoride membrane (Bio-Rad) and probed for the HNE-modified protein by incubating with the primary antibody (10 000:1; Alpha Diagnostic International), followed by incubation with hors eradish peroxidase–conjugated secondary antibody (20 000:1; Santa Cruz Biotechnology). Blots were visualized using the ECL system (Amersham Biosciences). Equal protein loading was confirmed by measuring α-tubulin.

Cell Count and Statistical Analysis

In each HNE-, 8-OHdG-, Iba-1, and GFAP-stained section, the stained cells in 3 predefined areas (0.25 mm2) were counted. Values presented in this study are expressed as mean±SEM. One-way ANOVA followed by post hoc Fisher protected least significant difference test was used to determine the significance of differences in various indexes among the different groups. A P value <0.05 denoted the presence of a statistically significant difference.

Results

Physiological Parameters

Measurement of various physiological parameters and rCBF revealed no significant differences between the edaravone and control groups during the whole process of ischemia and reperfusion.

Infarct Volume and Neurological Deficit

After ischemia/reperfusion injury, white-stained infarct area and severe neurological deficit were observed only in the operation group. In mice that received edaravone immediately after reperfusion, the infarct volume was significantly reduced to ≈60.13±4.83% (P<0.05) of the control group at 24 hours after reperfusion. The reduction of infarct volume at 72 hours and 7 days was 54.6±4.57% (P<0.01) and 47.98±2.52% (P<0.05) of the control group, respectively (Figure 1A and 1B). Furthermore, a significantly rapid and better functional recovery (as reflected by the neurological score) was noted, relative to the control group (P<0.05; Figure 1C). A significant reduction in infarct volume was also observed when edaravone treatment was delayed until 3 to 6 hours after reperfusion, but this reduction was not evident when the drug was administered 12 hours after reperfusion (Figure 1A and 1B). Moreover, edaravone significantly enhanced functional recovery when administered 3 to 12 hours after reperfusion compared with mice treated with the vehicle (Figure 1C).

Suppression of Lipid Peroxidation

Lipid peroxidation was assessed with anti-HNE antibody. In the sham operation group, HNE immunoreactivity was rarely observed. In the control group, immunoreactivity for HNE was detected as early as 3 hours after reperfusion at the ischemic core and penumbra area and was present in the cell bodies of neurons, with fainter straining in axons. The number of stained cells significantly increased at 12 hours of reperfusion and persisted until 72 hours. In the edaravone group, the number and the intensity of immunoreactivity in HNE-positive cells were significantly decreased from the early period after reperfusion compared with the control (Figure 2A and 2B).
Analysis of proteins extracted from the penumbra area showed a 34-kDa band on Western blotting using anti-HNE antibody in the operation group, whereas the band was not evident in the sham operation group. The accumulation of HNE-modified protein paralleled the immunohistochemical findings. The intensity of HNE-positive band increased at 12 hours of reperfusion and remained elevated until 72 hours in the control group. In the edaravone group, the intensity of the band decreased significantly from 3 hours after reperfusion (Figure 2C and 2D).

Suppression of Oxidative DNA Damage
We identified oxidative DNA damage by anti–8-OHdG antibody. No positive staining was detected in the sham operation group. After 3 hours of reperfusion, strong 8-OHdG immunoreactivity was evident in the nuclei of neurons.
located in the ischemic core in the control group. The 8-OHdG–positive cells were detected throughout the penumbra, and their numbers increased in a time-dependent manner. In comparison, nuclei of neurons in the edaravone group showed a weakly positive immunoreactivity for 8-OHdG since the early period after reperfusion (Figure 3A and 3B).

Effect of Edaravone on Microglial Activation
No active microglial cells were detected in the vehicle- or edaravone-treated sham operation group. In the control group, ramified Iba-1–positive microglia were first observed in the ischemic core from 3 hours after reperfusion, and their number notably increased at 12 hours. Such microglial activation was widely distributed and gradually increased to the penumbra until 7 days after MCA reperfusion. In the edaravone group, the number of microglia in the ischemic core evidently increased within 12 hours (Figure 4A and 4C), and after 24 hours of reperfusion, a significant reduction was noted in the penumbra area compared with the control group (Figure 4B and 4D).

Effect of Edaravone on iNOS Expression
The iNOS immunostaining was observed in endothelial cells and microglia throughout the penumbra area from 24 hours after reperfusion only in the operation group, and the intensity reached a peak level at 72 hours to 7 days after reperfusion. Although there was no significant difference in iNOS expression in endothelial cells between the 2 groups, a significantly weaker immunostaining was detected in the microglia of the edaravone group throughout most of the time series compared with the control group (Figure 5).

Effects of Edaravone on Nitrotyrosine Formation
Double immunostaining studies showed no coexistent staining for nitrotyrosine and microglia at any time point after reperfusion (data not shown). Whereas coexistent staining for nitrotyrosine and neuron marker was noted, such pattern was similar in the edaravone group and vehicle group (data not shown). Only a few nitrotyrosine-immunoreactive glia were detected in the corpus callosum area of the sham-operated mice treated with the vehicle or edaravone. In the control
The major findings of the present study were that edaravone suppressed the early accumulation of lipid peroxidation products and oxidative DNA damage and eliminated the sequential inflammatory responses. Moreover, even when edaravone administration was delayed until 6 hours after reperfusion, it significantly improved neurological function and reduced the infarct volume.

After ischemia/reperfusion injury, oxygen free radicals contribute to ischemia/reperfusion-induced damage in a consecutive 2-phase pattern, an immediately occurring direct cytotoxic damage and a posts ischemia/reperfusion inflammatory injury. To our knowledge, this is the first report that describes the therapeutic effects of edaravone on oxidative damage at an early period after ischemic insult and describes the relationship between edaravone and the inflammatory response.

We reported previously the distribution and alteration of HNE-modified protein after ischemia/reperfusion injury. HNE-modified proteins were detected in neurons in the infarct zone after 3 hours of reperfusion. HNE, as a marker of lipid peroxidation, seems to be useful to follow the progress of lipid peroxidation at cellular level after ischemic injury. The present study demonstrated that accumulation of HNE-modified protein and 8-OHdG was suppressed significantly by edaravone from the early period (3 hours) after reperfusion, and these effects lasted until 7 days. Our results clearly indicate that the neuroprotective role of edaravone was mediated via its antioxidant effect through the suppression of lipid peroxidation and oxidative DNA damage. Recently, Zhang et al demonstrated that the combination therapy of edaravone plus tissue plasminogen activator significantly increased the survival rate of transient MCAO rat and reduced the infarct volume and hemorrhage, and that the neuroprotective effect of edaravone treatment was dependent on reduced accumulation of lipid peroxidation products. However, their study did not perform quantitative analysis or investigate the serial changes in accumulation of HNE-modified protein and 8-OHdG. To our knowledge, the present study provides the first evidence for the neuroprotective role of edaravone against accumulation of lipid peroxidation products and oxidative DNA damage in the early period of cerebral ischemia/reperfusion injury. Although HNE can highly react with sulfhydryl groups or histidine and lysine residues of proteins, we clearly detected a single band corresponding to HNE-modified protein with proteins ex-
tracted from the penumbra by Western blotting analysis. In the edaravone group, the intensity of the band decreased from 3 hours after reperfusion compared with the control group. Other studies are required to identify the protein modified by HNE and its altered biological functions.

In the present study, the other interrelated mechanism that might account for the neuroprotective effect of edaravone is its anti-inflammatory effect mediated by induction and inhibition of microglial activity, inhibition of iNOS activity, and nitrotyrosine production. Microglial activation is considered a rapid cellular response to cerebral ischemia. However, it is not clear at present whether the microglial response to injury is beneficial or harmful. Activated microglia support tissue repair processes by rapid removal of debris as well as secretion of neurotrophins, but they also induce cytotoxic mediators such as NO and inflammatory cytokines, which may contribute to the infarct progression in the postischemic period. Accordingly, pharmacological suppression of microglial activation after ischemia has attenuated the extent of cell death and tissue damage. In the present study, we demonstrated that edaravone increased the number of activated microglia within 12 hours in the ischemic core; however, it decreased the response of activated microglia from 24 hours in the penumbra after reperfusion. Because our previous present results showed that iNOS expression was not detected in microglia until 24 hours after reperfusion, we speculate that the increase in activated microglia population by edaravone treatment might exert a neuroprotective effect in the early postischemic period.

In the control group, iNOS immunoreactivity was observed in activated microglia at 48 hours to 7 days after reperfusion. However, in the edaravone group, iNOS immunoreactivity was rarely detected in microglia but was evident in endothelial cells. In addition, nitrotyrosine-positive astrocytes were observed in the control group, but only few were detected in the edaravone group. Many evidences, such as iNOS-deficient mutant mice and administration of iNOS inhibitors, support the hypothesis that inhibition of iNOS production is of value in the treatment of the late stage cerebral ischemia. The appearance of peroxynitrite (ONOO−)-mediated nitrotyrosine paralleled NO synthesis, demonstrated by induction of iNOS, which produces high, potentially toxic level of NO. Based on our results that edaravone reduced nitrotyrosine production in astrocytes, the possible scenario behind edaravone-induced reduction of the infarct volume includes suppression of microglial activation followed by induction of iNOS and inhibition of peroxynitrite production. These finding suggest that one neuroprotective mechanism for edaravone may involve inhibition of peroxynitrite production through reduction of iNOS activity. Together, the neuroprotective effects of edaravone may include part of its anti-inflammatory mechanisms.

Summary
In summary, we showed that edaravone provides neuroprotection in the integrated process of oxidative stress–induced injury. We demonstrated that edaravone has antioxidant properties resulting in the suppression of lipid peroxidation products and oxidative DNA damage as well as anti-inflammatory effects resulting in reduction of iNOS production. Both actions are important for the neuroprotective effects of edaravone in stroke. Our results are considered important for extension of the therapeutic time window in acute cerebral ischemia.

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