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 administrated 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

There is increased evidence for the contribution of oxidative stress to ischemia/reperfusion-induced damage in a consecutive 2-phase pattern. In addition to the direct cytotoxic effects of lipid peroxidation, oxidative DNA damage occurring immediately after ischemia/reperfusion, the burst of free radicals also induces the formation of inflammatory mediators through redox-mediated signaling pathways, leading to postischemia/reperfusion inflammatory injury. Therefore, the control of these 2 impacts of oxidative damage is important to achieve neuroprotection.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a potent scavenger of hydroxyl radicals, has been approved by the Japanese health authorities as a neuroprotective agent for the treatment of acute cerebral infarction since 2001. Recent results indicate that when edaravone is used alone within 72 hours after the onset of stroke, it significantly reduces the infarct volume, and the outcome data indicate a sustained benefit during 12-month follow-up period. In a rat transient cerebral ischemia model, edaravone prevented cortical edema, reduced the infarct volume, and improved neurological deficits. Moreover, Zhang et al demonstrated that the combination therapy of edaravone plus tissue plasminogen activator was useful for the extension of the therapeutic time window in a rat transient cerebral ischemia model. Although the neuroprotective mechanism of edaravone has been investigated widely, to our knowledge, there is no report that describes the therapeutic effects of edaravone on oxidative damage at an early period after ischemic insult and the relationship between edaravone and the inflammatory response.

In the present study conducted in a mouse transient focal ischemia model, we tested the hypothesis that edaravone can suppress the accumulation of lipid peroxidation and oxidative DNA damage, and that it can provide neuroprotection by suppression of the inflammatory pathway of microglial activation, leading to inhibition of inducible NO synthase (iNOS) activity.
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 after reperfusion and 12 hours later. Ischemia was induced by the intraluminal vascular plex method (Vectastatin; Vector Laboratories) as described previously.8 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 MCA occlusion (MCAO). Mice of the therapeutic assessment group (n = 12 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 delayed to 3, 6, or 12 hours after reperfusion to assess the therapeutic time window. Ischemia was induced by the intraluminal vascular occlusion method as described previously.8

Immunohistochemistry

Immunohistochemistry was performed on 20-μm-thick free-floating coronal sections, which were prepared as described previously.8 After incubation in 3% H2O2 followed by 10% block ace in 0.1 PBS, the sections were strained 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.8

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. An anti-rabbit IgG secondary antibody was used to demonstrate the neuronal marker and an anti-mouse IgG secondary antibody was used to demonstrate the microglial or astrocytal marker. 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.

SDS-PAGE and Immunoblotting

Proteins were extracted from the penumbra area and measured as described previously.8 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 horseradish 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 staining in axons. The number of strained 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
group, nitrotyrosine-positive cells, characterized by short and thin processes, were detected in the penumbra area from 72 hours after reperfusion. At 7 days after reperfusion, the cells increased in number and showed stronger staining intensity. In the edaravone group, a low number of weakly stained cells were detected. Double immunostaining for nitrotyrosine and the astrocyte marker GFAP determined the coexistence of both substances in part of glial processes (Figure 6A). Next, we evaluated the effect of edaravone on the ratio of nitrotyrosine-positive cells to GFAP-positive cells. The ratio of nitrotyrosine-positive cells to GFAP-positive cells was not significantly different in the 2 sham operation groups. However, after the ischemia/reperfusion injury, the ratio was significantly reduced in the edaravone group compared with the control group (edaravone 44±9%, control 73±4%; edaravone 53±11%, control 81±5%, at 72 hours and 7 days after reperfusion, P<0.01 and P<0.05, respectively; Figure 6B).

Discussion

In the present study, we evaluated the effects of edaravone in a mouse model of focal cerebral ischemia/reperfusion injury. 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 postischemia/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-

Figure 5. INOS staining in representative control group and edaravone group. Shown are 3 hours (a and e), 24 hours (b and f), 72 hours (c and g) and 7 days (d and h) after reperfusion. In the control group, INOS immunoreactivity was detected in the endothelial cells and microglia at the penumbra, whereas INOS immunoreactivity was observed weakly in the microglia of the edaravone group. Bar=20 μm.

Figure 6. A, Double immunofluorescent staining of nitrotyrosine and GFAP. Shown are the corpus callosum area of the sham operation group (Aa, Ag, and Am for the control group and Ab, Ah, and An for the edaravone group), and at the penumbra of 72 hours (Ac, Ai, and Ar for the control group and Ad, Aj, and Ap for the edaravone group), and 7 days (Ae, Ak, and Aq for the control group and Af, Al, and Ar for the edaravone group) after reperfusion. Top, Nitrotyrosine immunoreactivity (red) in glial-like cells. Middle, GFAP immunoreactivity (green) shows the distribution of astrocytes. Bottom, Coexistence (yellow) of nitrotyrosine and GFAP in glial prolongations. Bar=20 μm. B, Ratio of nitrotyrosine-positive cells to GFAP-positive cells. Values are expressed as mean±SEM; n=3 each group. *P<0.05; **P<0.01 vs control.
The appearance of peroxynitrate (ONOO−)-mediated nitrotyrosine paralleled NO synthesis, demonstrated by induction of iNOS, which produces high, potentially toxic level of NO.\textsuperscript{14}\textsuperscript{15}\textsuperscript{16} 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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