Edaravone Inhibits JNK-c-Jun Pathway and Restores Anti-oxidative Defense after Ischemia-Reperfusion Injury in Aged Rats

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Edaravone, a potent antioxidant, is currently being used in the management of acute ischemic stroke in relatively high-aged populations. Mitogen activated protein kinase (MAPK) pathways have been shown to play important roles in neuronal cell death. We examined the role of MAPK pathways and the effect of treatment with edaravone in the brain after cerebral ischemia-reperfusion (I/R) injury in a bilateral carotid artery occlusion (BCAO) model with ischemia for 85 min followed by reperfusion for 45 min in aged rats. Western immunoblotting, immunostaining, enzyme-linked immunosorbent assay (ELISA), spectrophotometry, terminal deoxynucleotidyl transferase nick end labeling (TUNEL) and triphenyl tetrazolium chloride (TTC) staining were performed to evaluate various proteins in the homogenate, c-Jun NH2-terminal kinase (JNK) in the tissue sections, protein carbonyl, glutathione peroxidase (GSHPx), apoptosis and infarct size, respectively. Our results showed that I/R injury resulted in a reduction of GSHPx, but protein carbonyl content and inducible nitric oxide synthase were increased. The activation of JNK and its downstream molecule c-Jun was significantly increased after injury, whereas the activities of p38 MAPK and extracellular-regulated kinase 1/2 were slightly but not significantly increased. Edaravone (3 mg/kg, i.v.) treatment significantly reduced all of these changes. Our findings suggest that the JNK pathway differentially mediates neuronal injury in aged rats after BCAO, and edaravone treatment significantly reduces the neuronal damage after I/R injury by inhibiting oxidative stress and the JNK-c-Jun pathway with concomitant inhibition of overall MAPK activity in the brains of aged rats.

Key words cerebral ischemia-reperfusion; edaravone; mitogen-activated protein kinase; aged rat; neuronal damage; oxidative stress

The incidence of stroke is significantly higher in the geriatric population, with 75% of strokes occurring over the age of 65.1,2 Oxidative insult after ischemia-reperfusion (I/R) injury enhances oxidative modification of lipids and proteins, and modulates apoptotic signaling pathways. Oxidative stress leads to a rise in protein carbonyl content, which increases with age,3,4 and thus aged rats are more susceptible to ischemic injury. A high level of glutathione peroxidase (GSHPx), an important antioxidant enzyme, confers greater protection against oxidative stress than either superoxide dismutase or catalase alone, and GSHPx is usually reported to decrease after ischemic insult.5

Cerebral ischemia for 85 min followed by reperfusion for 45 min results in generation of oxidative stress.6 Free radicals have been shown to be associated with both ischemic and post-ischemic injury to the brain.7 Neuronal damage is enhanced after I/R injury and may be manifested as either apoptosis or necrosis of neurons.8 Several studies have demonstrated activation of mitogen activated protein kinase (MAPK) pathways in the enhancement of neuronal cell death in the cortex and hippocampus regions after global cerebral ischemia.9 Some reports also suggest that hydrogen peroxide (H2O2) and nitric oxide (NO) activate MAPK, which may result in the subsequent elevation of NO.10

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a potent free radical scavenger, is currently being used in the management of acute ischemic stroke.11,12 Edaravone has potent hydroxyl radical scavenging properties and reduces nitric oxide production dose dependently.13 Its ability to prevent lipid peroxidation is comparable to that of ascorbic acid and α-tocopherol.14 Furthermore, it has recently been reported to confer protection against ischemia-induced neuronal damage in the neonatal rat brain.15 However, the effect of edaravone on the MAPK pathways after global cerebral ischemia is still obscure. In the present study, we have investigated the effect of edaravone on I/R injury-induced neuronal damage in aged rats, with a focus on the MAPK family members c-Jun NH2-terminal kinase (JNK), extracellular regulated kinase 1/2 (ERK1/2) and p38 MAPK, and c-Jun, which is the key transcription factor of the activator protein (AP)-1 complex.

MATERIALS AND METHODS

Animals Male Sprague–Dawley rats (Charles River Japan Inc., Kanagawa, Japan) of about 24 months of age, weighing 500—540 g, were used in the present study. Experiments were performed in strict accordance with the guidelines for animal experimentation of our institute. The rats were divided into three groups for the study: Sham group (without any treatment; n=6), Vehicle group (rats were treated with saline after bilateral carotid artery occlusion (BCAO); n=6), and Edaravone group (edaravone was dissolved in saline and administered intravenously at a dose of 1.5 mg/kg at 5 min and 35 min post BCAO; n=6).

BCAO The experimental model was adopted from a previous report.16 During the surgical procedure, the body temperature of the animal was maintained at 37.5 °C with a heat-
ing pad (Model: TP-401, Gaymar Industries Inc., New York, NY, U.S.A.). Anesthesia was maintained with pentobarbital at a dose of 30 mg/kg. Cerebral ischemia was produced by the occlusion of both the right and left common carotid arteries exposed through a middle skin incision using aneurysm clips for 85 min; cerebral ischemia was confirmed by the change in color of the eyeball from red to white. At the end of the ischemic period, the carotid arteries were de-clamped to allow blood reperfusion for 45 min. All rats were decapitated under anesthesia and then the whole brain was excised and used for analysis.

**Measurement of Infarct Size** After I/R injury, infarct size was measured macroscopically as follows: The brain tissue was removed and sliced into five 2.0-mm-thick coronal sections. Brain slices were stained with 2% 2,3,5-triphenyl tetrazolium chloride (TTC) in phosphate buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 2.9 g Na2HPO4· 12H2O, 0.2 g KH2PO4 in 1000 ml of distilled water, pH 7.4) at 37 °C for 30 min and then fixed with 10% formalin in saline (pH 7.2) for 24 h.17) The viable cells were reddish brown in color, whereas the infarcted area was dull yellow in color. Based on the color difference, the infarct area was identified and calculated, and was expressed as a percentage. The investigator who evaluated the slides was blinded to the status of experimental groups.

**Brain Tissue Homogenate** The whole brain was removed, weighed, and immediately chilled in liquid nitrogen. Western blotting analysis was carried out using brain tissue samples homogenized in lysis buffer [50 mm Tris–HCl (pH 7.4), 200 mm NaCl, 20 mm NaF, 1.0 mm Na3VO4, and 1.0 mm sodium EDTA] in phosphate buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 2.9 g Na2HPO4· 12H2O, 0.2 g KH2PO4 in 1000 ml of distilled water, pH 7.4) at −80 °C until use. For assessment of GSHPx activity and protein carbonyl content, brain tissue was suspended in cold 0.05 M PBS containing 1.15% (w/v) KCl (9 ml per 1 g of wet tissue), after washing with 0.85% NaCl, and then homogenized at 0 °C using a Polytron PT 10-35 homogenizer (Kinematica AG, Littau, Switzerland). Total protein concentration of samples was measured using the bicinchoninic acid (BCA) protein assay using bovine serum albumin (BSA) as standard.

**GSHPx Activity Measurement** GSHPx activity was estimated according to the method of Wendel.18 Briefly, an aliquot of brain homogenate (0.4 mg of protein) in 0.05 M PBS containing 1.15% (w/v) KCl was mixed in a microplate with 230 µl of coupling solution (containing 33.6 mg of di-sodium EDTA, 6.5 mg of Na2S, 30.7 mg of reduced glutathione, 16.7 mg of NADPH, and 100 units of glutathione reductase in 100 ml of 50 mm Tris–HCl pH 7.6). The volume then was adjusted to 260 µl with 0.05 M PBS. Kinetic decay of NADPH fluorescence (Ex. 355 nm/Em. 465 nm) was measured after the addition of 40 µl of 1 × 10−3 M H2O2 as the substrate using a micro plate spectrophotometer (Labystem Fluoroskan Ascent CF, Osaka, Japan).

**Protein Carbonyl Measurement** The protein carbonyl in brain tissues homogenate was measured by the enzyme-linked immunosorbent assay (ELISA) method.19 Briefly, BSA oxidized by CuSO4/H2O2 (3 mm/5 mm) was used as the protein carbonyl standard. The carbonyl content of standard oxidized BSA was determined by a colorimetric method reported previously.20 The tissue homogenate was centrifuged at 3500 rpm for 10 min to remove the debris. Then the supernatant was diluted 2 fold with PBS and incubated with 10% streptomycin sulfate (9 : 1, v/w) for 15 min at 4 °C. After centrifugation at 10000 rpm for 10 min, the protein concentration in the supernatant was measured by the BCA method using BSA as a standard and then the protein concentration was adjusted to 1 mg/ml with PBS. The samples (500 µl) were reacted with 10 mm 2,4-dinitrophenylhydrazine (DNPH) in 2.5 x HCl (100 µl) at room temperature for 1 h. Proteins in samples and oxidized BSA were precipitated with 20% trichloroacetic acid (TCA, 500 µl) and the protein concentration was measured again and adjusted to 4 µg/100 µl. The standard curve for ELISA was prepared using oxidized BSA diluted with 40 µg/ml BSA at a defined ratio (0–40%). Aliquots (100 µl) of test samples and standards (4 µg of protein) were loaded into a 96-well immunoplate and incubated overnight at 4 °C. The plate was washed with PBS containing 0.1% Tween 20 (PBST) and then incubated with blocking buffer (1% BSA in PBST) for 2 h at room temperature. The samples were further incubated with primary antibody i.e., mouse anti-dinitrophenyl (DNP) IgE, (Sigma, St. Louis, MO, U.S.A.) for 4 h at 37 °C, washed with PBST and then incubated with secondary antibody, i.e., rat anti-mouse IgE, (Southern Biotechnology Associates Inc., Birmingham, AL, U.S.A.) for 1 h at 37 °C. The peroxidase reaction was performed by the addition of 100 µl of 3,3' ,5,5' tetramethyl benzidine (Sigma, St. Louis, MO, U.S.A) and stopped by adding 100 µl of 0.18 M H2SO4. The absorbance was measured at 450 nm using a micro plate reader (Model 550, Bio-Rad, CA, U.S.A.).

**Protein Analysis by Western Blotting** In order to study the role of the MAPK pathway and inducible nitric oxide synthase (iNOS) in global cerebral I/R injury, Western blotting of rat brain homogenates was performed with antibodies highly specific for the dual phosphorylated active forms of JNK, ERK, p38 MAPK and c-Jun, and iNOS. MAPK activation was quantified by normalizing the phospho-MAPK expression level with the total MAPK expression in the same samples, and iNOS expression was normalized with the GAPDH expression in the same sample. The MAPK activation in the Sham group was taken as 1 arbitrary unit. Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters. The filters were blocked with 5% non-fat dried milk in TBS-T (20 mm Tris, pH 7.6, 137 mm NaCl, and 0.5% Tween 20) for 1 h at room temperature. Anti-phospho JNK rabbit polyclonal antibody, anti-JNK rabbit polyclonal antibody, anti-phospho ERK1/2 mouse monoclonal antibody, anti-ERK1/2 rabbit polyclonal antibody, anti-phospho c-Jun rabbit polyclonal antibody, anti-c-Jun rabbit polyclonal antibody, anti-phospho p38 MAPK rabbit polyclonal antibody, anti-p38 MAPK rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA, U.S.A.), anti-iNOS mouse monoclonal antibody (Sigma), and anti-GAPDH goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were used at a dilution of 1 : 1000. After incubation with primary antibody, bound antibody was visualized with the respective horseradish peroxidase (HRP)-coupled secondary antibody (Santa Cruz) and chemiluminescence developing agents (ECL Plus, Amer sham, Piscataway, NJ, U.S.A.).

**Histopathology** a) Hematoxylin and Eosin (H-E) Staining: After 85 min of ischemia and 45 min of reperfusion, the brains were removed and fixed in 10% formalin. The brains
were then embedded in paraffin and representative coronal sections (6 μm thick), which included the dorsal hippocampus, were obtained using a rotary microtome. Tissue sections were stained with H-E.

b) Terminal Deoxynucleotidyl Transferase Nick End Labeling (TUNEL) Assay: Paraffin-embedded sections of brain tissue were deparaffinized and dehydrated in a descending alcohol series and then incubated in a 20 mg/ml solution of proteinase K, washed with PBS, incubated with 3% H2O2 for 5 min, and washed with PBS again. TUNEL staining was performed as specified in a TUNEL kit (Takara Bio, Shiga, Japan). Sections were mounted and examined using light microscopy. For each animal, five sections were scored regionally for TUNEL-positive cells located in both the cerebral hemispheres.

c) Immunohistochemistry for Phospho-JNK: Formalin-fixed, paraffin-embedded tissue sections were used for immunohistochemical staining. After deparaffinization and dehydration, the slides were washed in Tris-buffered saline (TBS; 10 mmol/l Tris HCl, 0.85% NaCl, pH 7.5) containing 0.1% BSA. Endogenous peroxidase activity was quenched by incubating the slides in a solution of 0.6% H2O2 in methanol. To perform antigen retrieval, the sections were pretreated with trypsin for 15 min at 37°C. Blocking was done with normal serum. After overnight incubation with primary antibody (phospho-JNK rabbit polyclonal antibody) at 4°C, the slides were washed in TBS buffer, and then HRP conjugated normal serum. After overnight incubation with primary anti-

**Statistical Analysis** Data are presented as means±standard error of the mean (S.E.M.). Statistical analysis of differences between the groups was performed by one-way analysis of variance followed by Tukey’s method. Correlations between groups of values were evaluated by calculating the best fit based on least-squares regression analysis and the coefficient of correlation (r) was determined. Differences were considered significant at p<0.05.

**RESULTS**

**Oxidative Stress after BCAO** The protective effect of edaravone against cerebral oxidative damage induced by I/R in rats was studied by assessing the oxidative inactivation of GSHPx enzyme. I/R injury resulted in a reduction of GSHPx activity by 37±2.6% compared to that in the untreated Sham group. The loss of GSHPx activity due to I/R was almost completely prevented by treatment with edaravone (Fig. 1A). Oxidative modification of proteins is accompanied by the generation of protein carbonyl derivatives. Protein carbonyl, a marker of protein oxidation in brain homogenates, increased to 247±5% in our model of I/R injury after BCAO and dropped to 110.6±4.5% in animals treated with edaravone at a dose of 3 mg/kg compared to the level in the Vehicle group (Fig. 1B). By measuring the expression of iNOS, an enzyme responsible for the formation of NO, the ability to produce NO was evaluated. Western blotting analysis was carried out using an antibody against iNOS. Densitometric analysis of iNOS normalized by GAPDH revealed a 2.96-fold increase in the expression of iNOS after I/R injury, and this increase was significantly attenuated to 2.21-fold in the group treated with edaravone (Fig. 1C).

**Activation of MAPK Pathways** We studied the activation of JNK, c-Jun, ERK and p38 MAPK after BCAO for 85 min followed by 45 min of reperfusion in rats. I/R injury significantly induced the activation of JNK and c-Jun in the Vehicle group (Figs. 2A, B). JNK activation was increased by 2.22±0.14 fold in the Vehicle group as compared to the Sham group (p<0.01), but only by 1.29±0.07 fold in the Edaravone group (Fig. 2A). c-Jun activation was significantly increased by 2.01±0.04 fold in the Vehicle group, but only by 1.19±0.05 fold in the Edaravone group (Fig. 2B). ERK 1/2 activation increased by 1.3±0.03 fold in the Vehicle group compared to the Sham group, but this effect was insignificant. In the Edaravone group, the activation of ERK1/2 activity was only 1.18±0.03 fold (Fig. 2C). The activation of p38 MAPK increased by 1.27±0.05 fold in the Vehicle group as compared to the Sham group and by 1.04±0.04 fold in the Edaravone group (Fig. 2D). As shown in Figs. 2E and F, the immunostaining for phospho-JNK in the hippocampus CA1 and cortex region was decreased in the Edaravone group compared to the Vehicle group.
Histological Findings of Neuronal Damage after BCAO

Characteristic histological features were seen in the rat brain after 85 min of ischemia and 45 min of reperfusion. Histo-pathologic changes were obvious in all ischemia-lesioned animals (Fig. 3). We observed a significant increase in TUNEL-positive cells in the Vehicle group (0.51 ± 0.03%; a 3.2-fold increase) after I/R injury, and this increase was inhibited in the Edaravone group (0.22 ± 0.02%) (Fig. 4A).

In addition, edaravone was found to affect infarct size, as shown in Fig. 4B. Edaravone caused an 8% decrease in infarct size as compared to that in the Vehicle group (p<0.01).

DISCUSSION

The incidence of stroke rises markedly with age. However, limited studies have addressed this issue in experimental animal models of stroke. Ischemic injury may be more lethal in older individuals, since the susceptibility of neurons to ischemia increases with age.1,2) We found that the administration of edaravone immediately after prolonged ischemia for 85 min was effective in preventing the neuronal damage observed in our model in aged rats. Early initiation of the treatment and re-administration of the drug after 30 min proved beneficial in the present study. Edaravone protects against I/R injury within a therapeutic time window,21) and edaravone treatment initiated early in patients with acute cerebral infarction and neurological deficits also leads to marked improvement and recovery22) and hence the protocol used here may simulate the actual clinical situation.

We observed a significant reduction in infarct size after edaravone treatment. Edaravone at a dose of 3 mg/kg has been reported to reduce infarct size in a model of focal ischemia in adult rats7,23) and to decrease the hydroxyl radical level at the ischemic border zone.24) Moreover, Mizuno et al. used the same dose administered twice in rats with middle cerebral artery occlusion, which resulted in a significant decrease in cerebral damage and the hydroxyl radical level.24) Furthermore, we demonstrated the effect of edaravone on the overall oxidative status after I/R injury. GSHPx is an antioxidant enzyme which facilitates the breakdown of superoxide to water and oxygen. GSHPx-1 knockout mice show increased infarct size with exacerbated apoptosis as compared to wild-type mice.25) On the other hand, mice overexpressing human GSHPx-1 isoform exhibit a reduction in infarct size by 48% after I/R injury.26) In the present study, the activity of GSHPx was significantly decreased after I/R injury, and this reduction was significantly attenuated by edaravone treatment. Our results are in accordance with the findings of Ichikawa and Konishi,16) and Xuejiang et al.9) NO, another potentially damaging free radical, mediates ischemic neuronal cell death.10) The level of iNOS expression in the Vehicle group was 2.96-fold that in the Sham group and was decreased significantly in the Edaravone group, as observed by Otani et al.21) Protein carbonyl derivatives, a marker of oxidative injury, have been reported to increase after ischemic insult to the brain.3) A substantial upsurge in protein oxidation was observed in the present study, as indicated by an increase in the protein carbonyl content (2.47-fold) in the Vehicle group as compared to the Sham group. Edaravone treatment resulted in an overall decrease in the reactive oxygen species.
and nitrogen species, and in protein oxidation in our model. In contrast, another report by Nagafuji et al. demonstrated a lack of iNOS participation in the ischemic brain damage,\(^{27}\) and thus these results should be interpreted cautiously.

Oxidative stress can trigger the activation of multiple signaling pathways which influence neuronal toxicity, including pathway involving increased phosphorylation of MAPKs\(^{28-30}\). JNK and p38 MAPK, which are activated by environmental stress and are closely associated with cell death, whereas ERK 1/2 is activated by growth factors and has an opposing effect on cell death. Several in vivo studies have demonstrated that the JNK, ERK1/2 and p38 MAPK pathways are activated after global ischemia in the hippocampus and cortex.\(^{9,28}\)

In the present study, we found that oxidative stress caused by BCAO for a period of 85 min followed by 45 min of reperfusion resulted in a marked increase in the activation of JNK (2.22 fold, \(p<0.01\)). Furthermore, immunostaining for phospho-JNK showed that it was increased in the Vehicle group in the hippocampus and cortex, and that this increase was attenuated in the Edaravone group, indicating that the neuronal injury occurring in our model is JNK-mediated. Slight increases in the activation of both ERK1/2 and p38 MAPK were observed in the Vehicle group compared to the Sham group, but these increases were not significant. The MAPK pathways are complex and are not yet fully understood: different cell types respond to the same stress in different ways, and different types of stress in the same cell activate different signaling pathways.\(^{31}\)

Moreover, evidence for the role of JNK is relatively stronger than that for p38 MAPK with respect to neuronal damage\(^{31}\) which supports the findings of the present study. On the other hand, the role of ERK1/2 in brain I/R injury is controversial.\(^{19,30,32}\) The activation of MAPK and the degree of its activation vary depending upon the type, degree and time of the stimulus. Presently, the molecular mechanisms of the significant activation of JNK and the moderate activation of other MAPKs are not known. Further studies will reveal the basis of the activation of MAPK kinases at different times after I/R, and the mechanism of the inhibition by edaravone of the MAPK pathways.

Among the various substrates phosphorylated by JNK that comprise the AP-1 transcriptional activator complex, c-Jun is a crucial component and has been shown to be a point of integration of numerous signals that can differentially affect its expression as well as its transcriptional activity.\(^{31}\) JNK activity is essential for the basal level of c-Jun expression and for c-Jun phosphorylation in response to stress.\(^{33}\) In the present study, the expression of phospho-c-Jun markedly increased in the Vehicle group, and this increase was prevented by edaravone treatment, indicating the involvement of c-Jun in the development of neuronal damage in the present study. Previously, N-acetyl cysteine, an antioxidant, was shown to significantly inhibit the JNK pathway and thereby to contribute to the protection against cerebral ischemic injury in rats\(^{24,35}\); however, it is not yet clear whether all antioxidants would act via the same mechanism. Very recently, activation of the JNK-c-Jun pathway was demonstrated to contribute to early...
phase apoptosis, in that study, pharmacological inhibition of JNK and overexpression of a dominant-negative form of c-Jun resulted in reduced neuronal damage, whereas p38 MAPK inhibitor failed to do so. Also, treatment with a specific inhibitor of JNK diminished the phosphorylation of c-Jun and thus provided protection from neuronal cell death.37) A neuroprotective effect of SP600125, a new inhibitor of JNK, against cerebral ischemia reperfusion injury was demonstrated in rats,38) and hence these kinds of inhibitors are important as future therapeutic options.

Global cerebral ischemia causes neuronal damage in regions that are specifically vulnerable to ischemia, such as the cerebral cortex and hippocampus.28) In the present study, we observed a 3-fold increase in total TUNEL-positive cells after 45 min of reperfusion following ischemic insult, primarily in the hippocampus, cortex, and thalamus regions of the brain, and this increase was significantly reduced in the Edaravone group. However, we consider that TUNEL staining detects both apoptotic as well as necrotic phenomena of cell death.39) Increased sensitivity of aged neurons to ischemia,40) decreased thresholds of stress-activated kinases41) and longer survival of aged rats in our model.

It may thus be concluded that edaravone significantly reduces infarct size, attenuates oxidative stress and prevents neuronal damage by inhibiting the activation of the JNK-c-Jun pathway in aged rats.

**Limitations** This study lacks information regarding whether all three MAPK pathways were activated in the injury-prone areas versus the whole brain, and experimentation with specific pharmacological inhibition of JNK may provide further clarification of this issue in our animal model.

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