Effects of Edaravone in Heart of Aged Rats after Cerebral Ischemia-Reperfusion Injury

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Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) has potent effects in the brain as a free radical scavenger in ischemia-reperfusion (IR) injuries. However, whether this free radical scavenger can prevent myocardial injury after cerebral IR is not clear. The aim of the present study was to investigate the effect of edaravone against oxidative damage in brain-to-heart signaling triggered by IR injury and its possible mechanism. In this study, the expression of glutathione peroxidase (GSHPx) and protein carbonyl content was examined to evaluate oxidative stress. The activation of mitogen-activated protein kinases (MAPKs) was also examined. Terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) analysis was performed to estimate cardiomyocytes cell death. After edaravone treatment there was a mild increase in activities of GSHPx in cardiomyocytes; however, there was a decrease in protein carbonyl content. p38 MAPK activity was inhibited by edaravone treatment in comparison with the vehicle group in myocardium. These results were further complemented by a significant reduction of TUNEL-positive cells in the heart sections. Our results demonstrate that edaravone provides ameliorative effects in the myocardium after cerebral IR injury by differentially modulating MAPKs’s activity, thus reducing the oxidative stress state.

Key words edaravone; brain-to-heart signaling; cerebral ischemia reperfusion; oxidative stress; myocardial injury

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a potent free radical scavenger, is currently used in the treatment of acute ischemic stroke.1–3 Its ability to prevent lipid peroxidation is comparable to that of ascorbic acid and α-tocopherol.4 Furthermore, it has been recently reported to confer protection against ischemia-induced neuronal damage in the neonatal rat brain.5

Mortality of ischemic heart diseases is significantly higher in old people than in young adults.6 There is very little information in the literature regarding age-related changes in myocardial function, injury, and inflammation during cerebral ischemia-reperfusion (IR) in vivo. Oxidative stress plays a critical role in IR.7–9 Reactive oxygen species (ROS) have the potential to injure cardiomyocytes, vascular cells, and endothelial cells directly,10,11 and may trigger an inflammatory cascade by inducing cytokine expression.8,12 Damage induced by ROS on intracellular and extracellular targets, such as membrane lipids, protein, and DNA clearly contributes to myocardial and/or apoptosis and organ dysfunction during myocardial IR.10,13 Normally, ROS are quickly inactivated by the antioxidative system; therefore the severity of oxidative tissue injury is determined by the balance between ROS production and intrinsic antioxidative capacity of the tissue.9–14 Aging may increase ROS production or enhance the toxic effects of ROS via impairment of antioxidative efficiency during myocardial IR.11,13 A devastating consequence of tissue reperfusion is the development of damage in organs uninvolved in the initial ischemic insult. From the standpoint of organ protection in the setting of IR, the heart is as important as the brain; because: a) the heart is a vital organ; and b) it is much less equipped in antioxidant defenses than other organs e.g. liver and intestines.16,17 In this study, we looked into the brain of IR-related myocardial damage in aged rats because this model simulates the clinical situation. Experimental data and clinical experience indicate that injury of the brain is often accompanied by secondary injury of the heart.18 In a previous study, we found that 45-min reperfusion of the brain after 85 min of ischemia triggers simultaneously an accumulation of protein carbonyl and mitogen-activated protein kinases (MAPKs) changes in the rat heart.19,20 In the present study, we focused on the effect of edaravone in the myocardial tissue of aged rat. We demonstrated that edaravone treatment significantly inhibits myocardial damage by regulating oxidative stress and the MAPKs pathway after cerebral IR.

MATERIALS AND METHODS

Animals Male Sprague–Dawley rats (Charles River Japan Inc., Kanagawa, Japan), 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: Sham group (n=6), Sham operation; Vehicle group (n=6), Vehicle-saline-treated rats with cerebral IR; Edaravone group (n=6), Edaravone dissolved in saline at a dose of 1.5 mg/kg and intravenously injected into jugular vein at 5 min and 35 min during reperfusion period in rats with cerebral IR.

Surgical Procedure The experimental model was adopted from Ichikawa et al.21 During the surgical procedure, the body temperature of animals was maintained at 37.5 °C with a heating pad (Model: TP-401, Gaymar Industries Inc., 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 and by using aneurysm clips to cause cerebral ischemia for 85 min and subsequently allow reperfusion for 45 min. All rats were decapitated under anesthesia then the whole heart was excised for analysis.

Tissue Homogenate The whole heart was removed,
was mixed in a microplate with 230 μg BSA diluted with 40 μg of BSA oxidized by CuSO₄/H₂O₂ (3 mM/5 mM) was used as the protein carbonyl standard. The carbonyl content of standard was determined by a colorimetric method using BSA as standard then the protein concentration was measured by bicinchoninic acid (BCA) protein assay using bovine serum albumin (BSA) as standard.

**Glutathione Peroxidase (GSHPx) Activity Measurement**

GSHPx activity was examined according to the method of Wendel. Briefly, an aliquot of heart homogenate (0.4 mg 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 disodium EDTA, 6.5 mg of NaN₃, 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). Total 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% BSA in PBST) for 2 h at room temperature. The samples (500 μl of test samples and standards (4—40%)) were reacted with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl (100 μl) at room temperature for 1 h. After centrifugation at 10000 rpm for 10 min, the protein concentration in the supernatant was measured by the BCA method using BSA as standard 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 M 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 at 4 °C overnight. The plate was washed with PBS containing 0.1% Tween 20 (PBST) 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.) at 37°C for 4 h, washed with PBST, then incubated with secondary antibody, i.e., rat anti-mouse IgE, (Southern Biotechnology Associates Inc., Birmingham, AL, U.S.A) at 37°C for 1 h. 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 by micro plate reader (Model 550, Bio-Rad, CA, U.S.A.).

**Protein Analysis by Western Blotting**

Western blotting was performed with antibodies highly specific for the dual phosphorylated active forms of c-Jun NH₂-terminal kinase-activated protein kinase (JNK), extracellular signal-related protein kinase (ERK1/2), p38 MAPK. MAPK activation was quantified by normalizing the phospho-MAPK expression level to the total MAPK expression in the same sample. 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) at room temperature for 1 h. 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 p38 MAPK rabbit polyclonal antibody, and anti-p38 MAPK rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA, U.S.A), were used at a dilution of 1:1000. After incubation with primary antibody, bound antibody was visualized with respective horseradish peroxidase-coupled secondary antibody (Santa Cruz) and chemiluminescence developing agents (ECL Plus, Amersham, Piscataway, NJ, U.S.A.).

**Terminal Deoxynucleotidyl Transferase Nick-End Labeling (TUNEL) Assay**

Paraffin-embedded sections of heart tissue were deparaffinized and dehydrated in a descending alcohol series then incubated in a 20-μg/ml solution of protease K, washed with PBS, incubated with 3% H₂O₂ for 5 min, and washed again with PBS. TUNEL staining was performed as specified in the kit (Takara Bio, Shiga, Japan). Sections were mounted and examined by light microscopy. For each animal, five sections were scored regionally for TUNEL-positive cells located in the samples. Only nuclei that were clearly located in cardiomyocytes were considered.

**Statistical Analysis**

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

**RESULTS**

**Effect of Edaravone on GSHPx Activity**

The protective effect of edaravone on IR was studied by examining the activity of GSHPx enzyme, an index of oxidative stress in our model. Although the activity of GSHPx in Vehicle group was decreased compared with Sham group, edaravone treatment caused a slight increase (Fig. 1A).

**Effect of Edaravone on Protein Carbonyl Content in Heart**

Oxidative modification of proteins is accompanied by the generation of protein carbonyl derivatives that is a marker of protein oxidation. The myocardial carbonyl content was markedly increased after IR in the Vehicle group and decreased in the edaravone treatment group (Fig. 1B), suggesting that edaravone could reduce cellular protein oxidative damage.
Effect of Edaravone on MAPK Pathways Activation
Cerebral IR increased p38 MAPK activity to 1.71±0.05 fold in cardiac tissue of Vehicle group compared with the Sham group. Edaravone treatment significantly reduced the activation of p38 MAPK to 1.23±0.047 fold (Fig. 2A). After IR, there was no significant change in JNK and ERK1/2 activation (Figs. 2B, C).

Effect of Edaravone on Myocardial Cell Death The result of TUNEL-assay showed that the increase in IR injury-induced TUNEL-positive cells in the myocardium was significantly inhibited by edaravone treatment (Figs. 3A, B). A significant positive correlation was found between the activation of p38 MAPK and total TUNEL-positive cells after IR injury (Fig. 3C).

DISCUSSION
In this study we used aged rats at 24 months of age. Aging may increase ROS production or enhance the toxic effects of ROS via impairment of antioxidative efficiency during IR, and older cells require more time to recover than those of younger animals.\textsuperscript{11,15} Canese et al. reported differences in severity and progression of brain ischemia in young and aged
Edaravone is lipophilic and rapidly accessible to intracellular space. It is clinically used for prophylactic treatment of acute stroke after cerebral infarction in Japan. The primary mechanism of this drug for cell protection is considered through direct scavenging of hydroxyl radical and through inhibiting lipoxygenase activity, which has been reported to play an important role in cardiac IR injury. A number of experimental and clinical studies have shown a protective effect of edaravone against ischemic injury in brain and liver even when administered after the onset of ischemia. The mechanism of edaravone action is based on hydroxyl radical scavenging and subsequent inhibition of the lipid peroxidation chain reaction in the plasma membrane of endothelial cells, neurons, and glial cells. However, there have been no reports about its direct effects on cerebral IR-associated heart damage in aged rats. We investigated the effect of edaravone in heart after cerebral IR injury since injury to the brain is often associated with a secondary injury to the heart.

GSHPx is an important antioxidant enzyme that protects cells from oxidative stress. In the present study we investigated myocardial oxidative stress after cerebral IR in aged rats (Fig. 1A). The activity of GSHPx was found slightly increased in the Edaravone group compared with the Vehicle group. In our previous study, we found that GSHPx activity in the brain was significantly increased in the Edaravone group. Hence at this moment the results are not very clear and this needs further investigation. Protein carbonyl derivatives, a marker of oxidative injury, have been reported to increase after ischemic insult to the heart. Edaravone treatment decreased myocardial protein carbonyl content in rat experimental autoimmune myocarditis. Edaravone treatment resulted in an overall decrease in ROS and protein oxidation.

Regarding the site of intracellular ROS generation upon reperfusion, the mitochondria electron transport chain is considered a primary source. Intracellular ROS levels increase during reperfusion and the extent of this increase correlates closely with cell death.

MAPKs are key kinases in signal transduction pathways and regulate cell growth and death. There are several types of MAPKs. ERK1/2 is activated by mitogens and survival factors whereas JNK and p38 MAPK are stimulated by stress signals. In the present study, we found that activation of p38 MAPK in cardiac tissue is significantly increased after brain IR. Ma et al. demonstrated that myocardial IR, a real pathological stress to the heart, results in significant activation of p38 MAPK. They have also provided evidence that activation of p38 MAPK plays a key role in the signal transduction pathway mediating myocardial apoptosis after IR, and found that inhibiting p38 MAPK, which reduces myocardial apoptosis associated with p38 MAPK, significantly improves postischemic cardiac functional recovery. In addition, our study highlights for the first time that edaravone significantly reduces p38 MAPK activation, but there was no significant change in the activity of JNK and ERK1/2 in cardiac tissue after cerebral IR. Furthermore, myocardial cell death was inhibited in the edaravone-treated group. Taken together, our results provide direct evidence that inhibition of p38 MAPK and reduction of subsequent myocardial cell death can ameliorate heart damage after cerebral IR. It is therefore possible that edaravone may attenuate the generation and/or actions of ROS in the heart resulting from oxidative stress following cerebral IR.

In present study, we demonstrated that edaravone attenuates myocardial damage induced by cerebral IR in aged rats mainly by decreasing elevated levels of protein carbonyl content in myocardial tissues and decreasing p38 MAPK activation. Furthermore, edaravone treatment significantly inhibited cardiomyocytes cell death. These findings indicate that edaravone plays a critical role in protection of the heart form brain IR injury and suggest a new concept for therapeutic intervention in such patients particularly in the older population. Thus the constantly increasing aging population in this century requires substantial investigation to prevent effectively and manage common age-related disease. In conclusion, we report that edaravone directly enhances cardiomyocyte survival upon cerebral IR injury.

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