Edaravone protects the retina against ischemia/reperfusion-induced oxidative injury through the PI3K/Akt/Nrf2 pathway

YI-PIN XU, FANG HAN and JIAN TAN

Department of Plastic Surgery, Shanghai Tenth People’s Hospital, Tongji University, Shanghai 200072, P.R. China

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Abstract. Retinal ischemia/reperfusion (I/R) injury can occur as a result of a number of ocular diseases or ischemic events in the brain, leading to possible vision loss if not treated properly. The overproduction of reactive oxygen species is important in the process of I/R injury. Edaravone, a free radical scavenger, has been demonstrated to have a neuroprotective effect in cerebral ischemia; however, its effect against retinal I/R injury remains to be fully elucidated. Therefore, the present study investigated the effects of edaravone on the oxidative parameters, retinal inflammation and apoptosis induced by I/R injury, and treated photoreceptor-derived 661W cells with hydrogen peroxide (H2O2) and edaravone to examine the underlying mechanism.

For the in vivo study, oxidative parameters (malondialdehyde, DNA fragmentation, total antioxidant status, superoxide dismutase and glutathione) in the retina, retinal thickness, and apoptotic index in the ganglionic cell layer and inner nuclear layer were measured. For the in vitro study, the effects of edaravone or nuclear factor erythroid-2-related factor 2 (Nrf2) small interfering RNA or phosphatidylinositol 3-kinase (PI3K)/Akt inhibitors on cell viability, membrane integrity, levels of phosphorylated-Akt, Akt and nuclear Nrf2 of H2O2-treated 661W cells were examined. The results demonstrated that edaravone inhibited the oxidative injury in the retina induced by the retinal I/R procedure and increased retinal inflammation, and apoptosis. The results of the in vitro experiments demonstrated that edaravone effectively protected the viability and the membrane integrity of the H2O2-treated 661W cells via the phosphatidylinositol 3-kinase (PI3K)/Akt/Nrf2 pathway. These results indicated the potential protective effect of edaravone against retinal I/R injury and provided a novel explanation for the protective effects of edaravone.

Introduction

As an important sensory organ, the retina converts photon energy into electrical impulses and transmits them to the brain. As a result, the retina has a high metabolic function and requires a continuous blood supply, which is provided by the choriocapillaris and the central retinal artery (1). However, the high rate of blood flow makes the retina susceptible to ischemia/reperfusion (I/R) injury. Retinal I/R injury can result from a number of ocular diseases, including retinal vascular occlusion, anterior optic neuropathy, diabetic retinopathy and glaucoma (2-5). In addition, ischemic events in the central nervous system can also cause irreversible loss of neurons present in surrounding areas, including the retina. Retinal I/R injury usually results in retinal ganglion cell death due to their vulnerability to ischemia (6,7). The time interval for retinal ischemia to cause irreversible damage is ~1 h (8). It is widely accepted that I/R injury is caused by the increased generation of reactive oxygen species (ROS) during the process of I/R, including superoxide (O2·−), hydrogen peroxide (H2O2) and hydroxyl radicals (OH) (9,10). Excessive ROS can react with DNA, lipids and proteins, leading to DNA breakage, lipid peroxidation and protein inactivation.

Edaravone has been shown to be neuroprotective in cerebral ischemia and has been approved for the treatment of cerebral infarction (11). Previous investigations have found that edaravone can eliminate ·OH and other ROS, including O2·− and nitric oxide radicals, and inhibit H2O2-induced lipid peroxidation (12,13). Edaravone can also activate anti-oxidative enzymes, including superoxide dismutase (SOD), catalase and guaiacol peroxidase (14). These findings suggest that edaravone may be effective for the treatment of retinal I/R injury. However, few investigations have examined the potential of edaravone in the prevention or treatment of retinal I/R injury, with the exception of a previous study by Song et al., which indicated that edaravone protected the retina from I/R injury in rats through reducing oxidative stress and inhibiting apoptosis of retinal neurons (15). However, the mechanism remains to be elucidated, and a detailed understanding of the molecular events following I/R induced retinal damage can facilitate the development of relevant treatments.

In the present study, retinal I/R injury was induced in rats and the effects of edaravone on oxidative parameters, including malondialdehyde (MDA), DNA fragmentation, total...
antioxidant status (TAS), SOD and glutathione (GSH) in the retina were investigated. Secondly, the retinal thickness and apoptotic index (AI) in the ganglion cell layer (GCL) and inner nuclear layer (INL) were measured to examine the protective effect of edaravone against I/R injury. To investigate the underlying mechanism, photoreceptor-derived 661W cells were treated with 1 mmol/l H$_2$O$_2$ to induce oxidative injury and with different concentrations of edaravone. The cell viability and levels of cellular lactate dehydrogenase (LDH) were examined, and involvement of the phosphatidylinositol 3-kinase (PI3K)/Akt kinase/nuclear factor erythroid-2-related factor 2 (Nrf2) pathway was investigated.

Materials and methods

Animals and drugs. Male Sprague-Dawley rats (8-12 weeks old, each weighing 220±50 g) were used in the present study. The animals were housed in the Hospital Animal Center of the Shanghai Tenth People's Hospital in polycarbonate cages at 25°C on a 12-h light/dark cycle, and were allowed free access to food and tap water. Ethical approval was obtained from the ethics committee of Tongji University (Shanghai, China) and performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (16). The rats were randomly divided into five groups: Control (rats received no treatment); Sham (rats received a sham retinal I/R surgery); I/R (rats received retinal I/R surgery and normal saline injection); I/R+edaravone (rats received a retinal I/R surgery and edaravone injection); Edaravone (rats received edaravone injection only). The edaravone injection was purchased from Nanjing Xiansheng Pharmaceutical Co., Ltd. (Nanjing, China). The edaravone and the normal saline were administered intra-peritoneally at a dose of 6 mg/kg body weight.

Retinal I/R injury procedure. The procedure used to induce retinal I/R injury was as previously described (17). Initially, the rats were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium (10 mg/kg). Following corneal anhesia with 0.4% oxybuprocaine hydrochloride and dilation of the pupil with 0.5% tropicamide and 0.5% phenylephrine, a 30-gauge needle was cannulated with the anterior chamber of the right eye. The other end of the needle was connected to a saline reservoir. Secondly, the pressure of the reservoir was decreased to the rat eye level and then divided into six groups: Control, H$_2$O$_2$ group (treated with 1 mmol H$_2$O$_2$ for 2 h), H$_2$O$_2$+25 µM Eda group (treated with 1 mmol H$_2$O$_2$ and 25 µM edaravone for 2 h), H$_2$O$_2$+50 µM Eda group (treated with 1 mmol H$_2$O$_2$ and 50 µM edaravone for 2 h), H$_2$O$_2$+100 µM Eda group (treated with 1 mmol H$_2$O$_2$ and 100 µM edaravone for 2 h) and 100 µM Eda group (treated with 100 µM edaravone for 2 h). Edaravone was purchased from Yuanye Biotech (Shanghai, China). Different doses of edaravone (25, 50 and 100 µM) were added to the culture 30 min prior to H$_2$O$_2$ treatment. In the second phase of the cell experiment, the 661W cells were divided into five groups: H$_2$O$_2$ group (treated with 1 mmol H$_2$O$_2$ for 2 h), H$_2$O$_2$+Eda+LY294002 group (treated with 1 mmol H$_2$O$_2$, 50 µM edaravone and 20 µM LY294002 for 2 h), H$_2$O$_2$+Eda+Nrf2 small interfering (si)RNA group...
(treated with 1 mmol H$_2$O$_2$, 50 µM edaravone and Nrf2 siRNA for 2 h), H$_2$O$_2$+Eda+triciribine group (treated with 1 mmol H$_2$O$_2$, 100 µM edaravone and 5 µM triciribine for 2 h). In the third phase of the cell experiments, the 661W cells were divided into six groups (control group and the same groups as in the second phase of the cell experiments). LY249002, purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany) was added to the medium to reach a final concentration of 20 µM. Nrf2-siRNA was purchased from Qiagen, Inc. (Valencia, CA, USA), including HP-validated siRNA and all stars Neg. siRNA AF488. The following sense and antisense sequences were used for Nrf2-siRNA forwards, 5'-GUAGAGACGGCAAGUGUAAdUdU-3' and reverse, 3'-dUdUCAUUCUUCGGUCUACAATT-5'. The 661W cells were transfected with Nrf2-siRNA for 72 h using HiPerFect transfection reagent according to the manufacturer's protocol (Qiagen, Inc.). Triciribine, purchased from Sigma-Aldrich; Merck Millipore, was added to the medium to reach a final concentration of 5 µM.

**Cell viability and LDH leakage assay.** Cell viability was determined using an MTT assay kit (Beyotime Institute of Biotechnology) similar to the method described by Bai et al (20). The cells were cultured in a 96-well plate (0.2x10$^5$ cells/ml) for treatment. Following treatment of the cells, 100 µl MTI solution (1 mg/ml in medium without serum and phenol red) was added to each well and the plates were incubated at 37°C for 3 h. Following incubation, the medium on top was removed and isopropanol was added to each well to dissolve the formazan crystals. Finally, the absorbance values were measured at 570 nm with an EIX-800 Micro elisa reader (BioTek Instruments, Inc., Winooski, VT, USA). Cell survival rates were determined as percentages of that of normal cells. The LDH leakage assay kit (CytoTox 96® non-radioactive cytotoxicity assay) was a product of Promega Corporation (Madison, WI, USA). The 661W cells were seeded into 96-well plates 12 h prior to treatment. Following treatment of the cells, 20 µl of the medium was transferred to a new 96-well plate to measure LDH activity, according to the manufacturer's protocol, as described by Chang et al (21).

**Western blot analysis.** Following treatment of the 661W cells, the cells were harvested and then re-suspended in lysis buffer (Cell lysis buffer for Western and immunoprecipitation; Beyotime Institute of Biotechnology). The samples were then centrifuged at 12,000 g for 10 min at 4°C and the supernatants were collected. The total protein levels were measured using a bicinchoninic assay (Roche Diagnostics GmbH). The protein (25 µg) was separated by 10% SDS-PAGE and transferred onto polyvinylidenedifluoride membranes (EMD Millipore). The membranes were blocked in 5% nonfat milk and then incubated with primary rabbit polyclonal antibodies against Akt (cat. no. sc-5298; 1:1,500), phosphorylated-Akt (p-Akt; cat. no. sc-293125; 1:2,000) and Nrf2 (cat. no. sc-365949; 1:2,000), which were bought from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), in 5 ml of 5% bovine serum albumin wash buffer (Beyotime Institute of Biotechnology) at 4°C overnight. The membranes were then washed and incubated with secondary anti-rabbit immunoglobulin G (1:2,000; cat. no. sc-2030; Santa Cruz Biotechnology, Inc.) in 5% milk wash solution for 1 h at 25°C. Digitized images of protein bands were quantitated using AlphaEaseFC™ software (version 4.0.0; Witec, Littau, Switzerland). β-actin was used as an internal control.

**Results**

**Oxidative parameters are increased by I/R and inhibited by edaravone.** The changes in oxidative parameters (MDA, DNA fragmentation, TAS, SOD and GSH) are exhibited in Table I. No significant alterations in these parameters were found in the Sham rats. The rats exposed to retinal I/R exhibited significant increases in MDA and DNA fragmentation, and significant decreases in TAS, SOD and GSH, compared with the control and sham group (P<0.05). However, these changes in the parameters in the I/R group were all significantly inhibited in the I/R+edaravone group: MDA and DNA fragmentation were decreased; and levels of TAS, SOD and GSH were increased (P<0.05). Compared with the control and sham groups, no significant changes in these parameters were observed in the edaravone group, with the exception of increased TAS (P<0.05).

**Edaravone inhibits the changes in retinal thickness, and AI in the GCL and INL induced by I/R.** The changes of retinal thickness and AI in the GCL and INL following treatment of edaravone are shown in Table II. No significant changes in these parameters were observed in the Sham rats (P>0.05). The rats exposed to retinal I/R exhibited a significant increase in retinal thickness, and increased AI in the GCL and INL, compared with those in the Control and Sham groups (P<0.05). These parameters in the I/R+edaravone group were significantly lower, compared with those in the I/R group (P<0.05). Compared with the Control and Sham group, no significant changes were observed in these parameters in the edaravone group (P>0.05).

**Edaravone protects cell viability and membrane integrity of H$_2$O$_2$-treated 661W cells.** The results of the cell viability analysis showed that: i) H$_2$O$_2$ treatment significantly decreased the viability of the 661W cells (P<0.05); ii) 25 µM edaravone had no significant effect on the cell viability of H$_2$O$_2$-treated 661W cells, however, 50 and 100 µM edaravone increased cell viability (P<0.05); iii) edaravone alone did not alter cell viability (Fig. 1A). The analysis of LDH leakage demonstrated similar results: i) H$_2$O$_2$ treatment significantly increased LDH leakage (P<0.05); ii) 25 µM edaravone did not significantly affect LDH leakage, however, 50 and 100 µM edaravone significantly decreased leakage (P<0.05); ii) edaravone alone did not affect LDH leakage (Fig. 1B).
Expression levels of p-Akt, Akt and nuclear Nrf2 in 661W cells are increased by edaravone. The expression levels of p-Akt, Akt and nuclear Nrf2 in cells were altered by oxidative stress and edaravone, as shown in Fig. 2. It was demonstrated that, following exposure of the 661W cells to $H_2O_2$ for 2 h, no significant changes were observed in the protein expression levels of p-Akt, Akt or nuclear Nrf2 (P>0.05). In the presence of 25 µM edaravone, the expression of p-Akt, Akt and nuclear Nrf2 remained unchanged, however, treatment with 50 and 100 µM edaravone led to significant increases in p-Akt/Akt and nuclear Nrf2 (P<0.05). Pretreatment with 100 µM edaravone alone also significantly increased the expression levels of p-Akt/Akt and nuclear Nrf2 in cells, compared with those in the Control (P<0.05).

Expression levels of p-Akt, Akt and Nrf2 in 661W cells are inhibited by Nrf2 siRNA or PI3K/Akt inhibitors. The expression of p-Akt, Akt (Fig. 3A) and Nrf2 (Fig. 3B) in cells were altered by Nrf2 siRNA and the PI3K/Akt inhibitor. As shown in Fig. 3A, treatment with Nrf2 siRNA had no significant effect on the p-Akt/Akt ratio (P>0.05), however, the PI3K inhibitor (LY294002) and Akt inhibitor (triciribine) significantly decreased the p-Akt/Akt ratio (P<0.05). For Nrf2 (Fig. 3B), the Nrf2 siRNA and the PI3K/Akt inhibitor significantly decreased its expression (P<0.05).

Nrf2 siRNA or PI3K/Akt inhibitors counter the protective effect of edaravone on cell viability and membrane integrity of 661W cells. The viabilities of 661W cells in the presence of PI3K/Akt inhibitors were significantly increased compared to those in the Control group (P<0.05).
inhibitors or Nrf2 siRNA are exhibited in Fig. 4A. Treatment of cells with Nrf2 siRNA, PI3K inhibitor (LY294002) or Akt inhibitor (triciribine) all eliminated the effect of edaravone on cell viability (P<0.05). The membrane integrities of 661W cells in the presence of Nrf2 siRNA or PI3K/Akt inhibitors are shown in Fig. 4B. Treatment with Nrf2 siRNA, LY294002 and triciribine all eliminated the effect of edaravone on LDH leakage (P<0.05).

Discussion

Retinal I/R injury is associated with various conditions, which can culminate in blindness if effective treatment is not provided (22). The retina consists of neurons, vasculature and glia, and each of these compartments can be affected in retinal I/R injury (23-25). The exact mechanism of cell death due to retinal I/R injury remains to be fully elucidated, however, it has been previously demonstrated that, in conditions of oxidative stress, retinal ganglion cells are damaged as a result of increased intracellular ROS and calcium influx (26). In the process of I/R, ROS are extensively generated in the early stage of reperfusion and can cause serious damage to various organs, including the brain and heart (27,28). It has also been demonstrated in multiple studies that the oxidative stress induced by ROS is key in the pathophysiological mechanisms involved in retinal I/R injury (29,30). As demonstrated in the results of the present study, the rats exposed to retinal I/R exhibited a significant increase in MDA and DNA fragmentation, suggesting that ROS caused the peroxidation of cellular lipid in addition to DNA oxidative damage. It also significantly
decreased the levels of TAS, SOD and GSH, indicating that the balance of oxidative/anti-oxidative in the retina was disturbed by I/R procedure. The production and accumulation of excessive ROS is considered to be important in the mechanism of I/R injury. ROS are the major free radicals in human body, including O$_2^·$, OH and H$_2$O$_2$. The nicotinamide adenine dinucleotide phosphate oxidase system, in conjunction with mitochondria, is a major site of ROS generation under H$_2$O$_2$ (31).

The overproduction of ROS can induce several inflammatory mediators, including interleukin 1-β and tumor necrosis factor-α, and apoptosis in the retina (32-34). As a result, inflammation and cell apoptosis are considered to be major causes of the pathological changes following I/R injury. As exhibited in Table II, the rats exposed to retinal I/R procedure exhibited significant increases in retinal thickness and apoptotic indices in the GCL and INL. The increase of retinal thickness indicated that the I/R injury caused retinal inflammation, whereas the increase of AI in the GCL and INL demonstrated cell apoptosis was induced by I/R.

Edaravone, a novel free radical scavenger, has been approved for the treatment of ischemic stroke in China and Japan. It is widely accepted that it produces neuroprotective effects by scavenging free radicals, and inhibiting lipid peroxidation and oxidative damage to cells (13). In the animal experiments performed in the present study, it was found that edaravone effectively attenuated the disruption of oxidative/anti-oxidative balance induced by retinal I/R injury. The oxidative parameters following I/R injury were all significantly inhibited by edaravone: MDA and DNA fragmentation were decreased; TAS, SOD and GSH were increased. Furthermore, edaravone significantly decreased retinal thickness and AI in the GCL and INL, indicating its protection against the retinal inflammation and cell apoptosis induced by I/R. These results were consistent with those in a study by Song et al (15). In this previous study, rats were injected with edaravone at 30 min prior to ischemia, following which retinal ischemia was induced by elevating intracocular pressure to 110 mmHg for 60 min and then treated with edaravone twice daily for 1 or 5 days post-I/R. An electoretinogram was recorded 5 days following reperfusion. It was concluded that edaravone lowered levels of MDA, increased SOD activity, and attenuated I/R-induced apoptosis of retinal neurons and suppressed I/R-induced reduction in a- and b-wave amplitudes of ERG. However, the molecular events following I/R-induced retinal damage were not examined. Understanding the underlying mechanisms may facilitate the development of relevant treatments.

To further examine the underlying mechanism of the protective effect of edaravone, the present study treated 661W cells, a mouse photoreceptor cell line, with H$_2$O$_2$ to produce oxidative stress, and examine the effect of edaravone on cell viability and injury. The results showed that edaravone dose-dependently enhanced cell viability, which was decreased by H$_2$O$_2$ treatment; it also dose-dependently protected cell integrity, which was impaired by H$_2$O$_2$, demonstrated by LDH leakage. Subsequently, the present study determined the effects of H$_2$O$_2$ and edaravone on the activation of Akt and expression of Nrf2. The results of the western blot analysis showed no significant changes in the protein expression levels of p-Akt, Akt or Nrf2 in the presence of H$_2$O$_2$, however, edaravone significantly increased p-Akt/Akt and Nrf2. The p-Akt/Akt ratio was not affected by treatment with Nrf2 siRNA, but was significantly decreased by the PI3K inhibitor (LY294002) or Akt inhibitor (triciribine). By contrast, the expression of Nrf2 was inhibited by Nrf2 siRNA and the PI3K/Akt inhibitors.

It has been revealed that the PI3K-mediated generation of 3'-phosphorylated phosphoinositide leads to the recruitment of Akt to the cell membrane, where it is phosphorylated by kinases, including phosphoinositide-dependent kinase-1, leading to the activation of Akt (35). Nrf2 is an important transcription factor in the coordinated expression of stress-inducible genes. Several studies have shown that Nrf2 can regulate the expression of phase-II detoxification and antioxidant response element, including glutathione synthase, hemooxygenase (HO)-1 and catalase, under oxidative stress, and can be activated by the PI3K/Akt pathway. For example, PI3K/Akt can facilitate the release of Nrf2 from the Keap1-Nrf2 complex, and enables it to translocate into the nucleus and induce phase II defense enzymes (36). A study by Hua et al (37) demonstrated that edaravone...
significantly induced the translocation of Nrf2 and HO-1 to the nucleus, and markedly increased components of the cellular antioxidant defense system, including GSH, SOD and HO-1, consistent with the present study. It is possible that the rearrangement of actin microfilaments and the increase of cellular Ca^{2+} are involved in the regulation of Nrf2 via the PI3K/Akt pathway (38).

In conclusion, the present study revealed that edaravone inhibited oxidative injury in the retina induced by retinal I/R, and reduced the increases of retinal inflammation and apoptosis. *In vitro* experiments demonstrated that edaravone effectively protected cell viability and the membrane integrity of the H_{2}O_{2}-treated 661W cells via the PI3K/Akt/Nrf2 pathway. These results indicate the potential protective effect of edaravone against retinal I/R injury and provide a novel explanation for the protective effects of edaravone.

References


