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European Journal of Pharmacology 516 (2005) 125 – 130

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Anti-apoptotic and neuroprotective effects of edaravone following transient focal ischemia in rats

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Received 20 January 2005; received in revised form 18 April 2005; accepted 22 April 2005
Available online 25 May 2005

Abstract

To investigate the effect of an antioxidant edaravone on the apoptotic process, we examined Bax and Bcl-2 immunohistochemical expression and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) reactivity. Rat focal ischemia models were prepared by 2 h transient middle cerebral artery occlusion. Edaravone or physiological saline was intravenously administered after reperfusion. After 24 h of reperfusion, infarction volume assessments, Bax and Bcl-2 immunohistochemistry and TUNEL staining were performed as well as neurological evaluation. Cortical cerebral blood flow was not statistically different between the treatment-groups. Edaravone-treated animals showed significantly improved neurological outcome. Total and cortical infarct volumes in the edaravone group significantly decreased. In addition, edaravone-treatment provided a significant reduction in the number of TUNEL-positive apoptotic cells, a decrease in Bax immunoreactivity and an increase in Bcl-2 expression within the peri-infarct area. Edaravone shows an excellent neuroprotective effect against ischemia/reperfusion brain injury through a Bax/Bcl-2 dependent anti-apoptotic mechanism.

Keywords: Edaravone; Free radical scavenger; Apoptosis; Focal ischemia; Neuroprotection

1. Introduction

Oxidative stress plays a central role among such various factors contributing to ischemic brain damage, as excitatory amino acid release, anoxic depolarization and specific gene expression, through reactive oxygen species that lead to lipid peroxidation and DNA damage (Chan, 1996). In addition, blood–brain-barrier disruption, mediated by oxygen free radicals, results in cerebral edema (Chan et al., 1984). Recent investigations have confirmed oxygen radical formation in the penumbral cortex during middle cerebral artery occlusion in rats (Morimoto et al., 1996; Solenski et al., 1997).

Oxidative stress resulting from reactive oxygen species production is also implicated in apoptosis. Although ischemic neuronal cell death had been traditionally interpreted by necrotic mechanisms, a role of apoptotic mechanisms has been recently proposed in acute neuronal cell death following brain ischemia (MacManus and Linnik, 1997). Several studies have suggested that apoptotic mechanisms were initiated at the molecular level in ischemic or post-ischemic neurons, especially those in the penumbral regions of focal ischemia (Charriaut-Marlangue et al., 1996; Li et al., 1997, 1998).

The endogenous antioxidant enzymes, such as superoxide dismutases (SODs), glutathione peroxidase and catalase, are known to scavenge reactive oxygen species, and neuroprotective effects of these free radical scavengers during ischemia/reperfusion have been variously demonstrated (Chan, 2001). However most antioxidant therapies

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doi:10.1016/j.ejphar.2005.04.036

have not shown any beneficial effects in clinical trials, despite their excellent efficiencies in animal models (Gilgun-Sherki et al., 2002). An antioxidant, 3-methyl-1-phenyl-pyrazolin-5-one (edaravone, MCI-186) is the first free radical scavenger which has provided clinical evidence for therapeutic effects on ischemic stroke, and has been applied to the clinical field in Japan since June 2001 (Tabrizch, 2000). Edaravone inhibits both hydroxyl radical generation and iron-induced peroxidative injuries, and reportedly has protective effects against ischemic damage (Abe et al., 1988; Kawai et al., 1997; Murota et al., 1990; Nishi et al., 1989; Oishi et al., 1989; Watanabe et al., 1988, 1994; Watanabe and Egawa, 1994). However it remains uncertain whether the protective effects of the antioxidant edaravone on cerebral ischemia are related to suppressing apoptotic pathways.

The purpose of the present study was to investigate the effect of edaravone on immunohistochemical expression of pro- and anti-apoptotic proteins as well as the numbers of apoptotic cells, labeled by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining, following transient focal ischemia in rats.

2. Materials and methods

2.1. Animal preparation and drug administration

All experimental protocols were approved by our Institutional Committee on Animal Research, and were carried out in accordance with the National Institutes of Health guidelines for animal use and care and use of laboratory animals. Adult male Sprague–Dawley rats ($n=14$ for each group) weighing 250–300 g underwent 2 h middle cerebral artery occlusion followed by 24 h reperfusion using an intraluminal suture technique, as described in detail previously (Katsumata et al., 2003; Nito et al., 2004). Briefly, the left common and external carotid arteries were carefully exposed and ligated using 4-0 silk suture through a ventral midline neck incision. A 4-0 nylon surgical thread, coated with silicon rubber, was transiently inserted into the left internal carotid artery (approximately 18 mm from the carotid bifurcation) for 2 h to induce transient focal ischemia. The caudal tail artery and left femoral vein were cannulated for continuous monitoring of mean arterial blood pressure, analysis for arterial blood gases and blood glucose (BG) level, and intravenous drug administration. Rectal and left temporal muscle temperatures were maintained at 37±0.3 °C during ischemia and for up to 60 min after reperfusion. Cortical cerebral blood flow in the left middle cerebral artery territory (5 mm lateral and 2 mm posterior to the bregma through a craniotomy of 1 mm in diameter) was measured during ischemia and up to 2 h after reperfusion using a Laser-Doppler flowmetry MBF3D (Moor Instruments Ltd, Devon, UK) in several animals ($n=4$, each), as described in detail previously (Nito et al., 2004). Sham-operated animals underwent the same procedures except for inducing focal ischemia. Animals were allowed free access to food and water after recovery from anesthesia for 24 h.

Animals were randomly divided into edaravone and vehicle groups ($n=14$, each). Edaravone (Mitsubishi Pharma Corporation, Tokyo, Japan) was dissolved in 1 N NaOH and titrated to pH 7.4 using 1 N HCl, and was diluted with physiological saline to the concentration of 3.0 mg/ml. Immediately and 30 min after reperfusion, rats received intravenous injections of 3.0 mg/kg of edaravone (edaravone group) or physiological saline (vehicle group).

2.2. Assessment of neurological scores

After 24 h of reperfusion, neurological symptoms in each rat were evaluated in a blind fashion using a neurological deficit score (grade 0–3) based on the detection of hemiparesis and abnormal posture ($n=14$, each), as described in detail previously (Katsumata et al., 2003; Nito et al., 2004). For the assessment of abnormal posture, rats were suspended by the tail, and forelimb flexion and body twisting were evaluated as 0 (normal), 1 (slight twisting), 2 (marked twisting), and 3 (marked twisting and forelimb flexion). For the evaluation of hemiparesis, the right hindlimb of each rat was extended gently with round tipped forceps and the flexor response was evaluated as 0 (normal), 1 (slight deficit), 2 (moderate deficit), and 3 (severe deficit).

2.3. Measurement of infarct volume and edema index

To assess infarct and edema volumes, animals were decapitated 24 h after reperfusion ($n=10$, each), and 20-μm-thick coronal frozen sections were cut on a cryostat (MICROM HM500-OM, Walldorf, Germany). Infarct areas were identified as distinct pale lesions on HE-stained sections obtained at a 500-μm interval.

The hemispheric and infarct areas of each section were traced using an image analyzing software Mac Scope 2.55 (Mitani Corporation, Fukui, Japan) on a Macintosh computer in a blind fashion. The infarct area in each HE-stained section was determined by subtracting intact area in the ischemic hemisphere from total area in the contralateral hemisphere to correct for brain edema (Swanson et al., 1990). Cortical and subcortical infarct areas were calculated separately. The infarct areas from each HE-stained section were summed and multiplied by the interval thickness to yield the total infarct volume. Edema index (%) was calculated as the following: (ipsilateral hemispheric volume − contralateral hemispheric volume) × 100 / contralateral hemispheric volume (Maier et al., 1998).

2.4. TUNEL staining and immunohistochemistry

Animals were transcardially perfusion-fixed with 4% paraformaldehyde 24 h after reperfusion ($n=4$, each), and 20-μm-thick coronal frozen sections at the level of the anterior commissure (bregma + 0.70 mm) were cut on a cryostat. The adjacent sections were stained with HE to confirm infarct border.

TUNEL staining was carried out using the in situ cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. TUNEL-positive cells were visualized and counterstained using an alkaline phosphatase substrate kit and nuclear fast red (Vector, CA, USA). Each procedure was followed by several rinses in 100 mM phosphate-buffered saline (PBS). A negative control was similarly performed except for omitting TUNEL reaction mixture. Only cells showing nuclear condensation/fragmentation and apoptotic bodies in the absence of cytoplasmic TUNEL reactivity were considered apoptotic, and cells demonstrating diffuse cytoplasmic staining were considered necrotic.

For immunohistochemistry, sections, blocked using 2% normal goat serum in PBS, were incubated in mouse monoclonal antibody against Bcl-2 at a dilution of 1:20 (M0887; Dako, Glostrup, Denmark) or rabbit polyclonal antibody against Bax at a dilution of 1:10 (PC66; Oncogene, MA, USA) at 4 °C for overnight. The sections were incubated in biotinylated anti-mouse or anti-rabbit secondary antibodies (Lab Vision, CA, USA) at room temperature for 1 h. Binding biotinylated antibodies were visualized using Vecstatin Elite Universal ABC kit and peroxidase substrate kit (Vector, CA, USA). These sections were counterstained using nuclear fast red. Each procedure was followed by several rinses in PBS. Blank staining was carried out in the same way as the above, except for eliminating the primary antibodies.

TUNEL-positive apoptotic cells were counted, and nuclear signals for Bcl-2 and Bax in the peri-infarct area were semi-quantified in randomly selected three microscopic fields (200 μm square, each) under a 100× magnification in a blind fashion ($n=4$, each). A four point rating scale was used for the semi-quantified analysis as the following: 0, none; 1, trace; 2, weak; 3, moderate; and 4, strong (Ay et al., 2001).

2.5. Statistical analysis

Statistical analysis was performed using StatView ver. 5.01 software (SAS Institute Inc., NC, USA) on a Macintosh computer. An unpaired *t*-test was used for comparisons in physiological parameters, cortical cerebral blood flow, infarct volume and edema index between the groups, and a Mann–Whitney *U*-test was carried out to compare neurological scores, number of TUNEL-positive cells and staining grades between the groups. Data are expressed as mean±S.D., and statistical significance was set at $P<0.05$.

3. Results

3.1. Physiological parameters

Physiological variables were within normal limits at any evaluating time points, and showed no statistically significant differences between the groups (Table 1).

3.2. Cortical cerebral blood flow

In both groups, middle cerebral artery occlusion resulted in an immediate cortical cerebral blood flow reduction to approximately 20% of baseline and remained unchanged throughout the ischemic period. Immediately after reperfusion, a short period of post-ischemic hyperemia (between 120% and 140%) followed by a gradual decrease in cortical cerebral blood flow was observed. Relative cortical cerebral blood flow showed no statistically significant differences at any time points between the vehicle group and the edaravone group (Fig. 1).

3.3. Neurological scores

Fig. 2 demonstrates the neurological scores regarding hemiparesis and abnormal posture ($n=14$, each). Hemiparesis scores were significantly improved in the edaravone group compared to the vehicle group ($P=0.031$), although abnormal posture scores were not statistically different between the groups.

3.4. Infarct volume and edema index

Fig. 3A shows total, cortical and subcortical infarct volumes in the groups. The total and cortical infarct volumes were significantly reduced in the edaravone-treated group compared to the vehicle group ($P=0.044$ and 0.025, respectively), however subcortical infarct volume was not statistically different between the groups. Fig. 3B indicates the edema index, which was also significantly improved in the edaravone group compared to the vehicle group ($P=0.043$).

3.5. TUNEL staining and immunohistochemistry for Bax and Bcl-2

The peri-infarct area was determined as just outside of the infarct border that was confirmed in the adjacent HE stained section. Representative microphotograph of TUNEL staining and immunohistochemistry for Bax and Bcl-2 are shown in Fig. 4. A moderate number of TUNEL-positive apoptotic cells were observed mainly in the peri-infarct areas in both groups. Bax and Bcl-2 immunoreactivities were detected mainly in cell bodies and processes of large pyramidal neurons within the peri-infarct areas in both experimental groups.

Fig. 5 shows semi-quantitative analysis of TUNEL staining and Bax/Bcl-2 immunohistochemistry. The number of TUNEL-positive cells was significantly reduced in the edaravone group compared to those in the vehicle group ($P=0.021$). Staining grades for Bax immunoreactivity in the peri-infarct areas were significantly reduced in the edaravone group compared to those in the vehicle group ($P=0.033$). Staining grades for Bcl-2 immunoreactivity

Table 1
Physiological parameters in the edaravone- and vehicle-treated animals

		pH	PCO_2 (mm Hg)	PO_2 (mm Hg)	MABP (mm Hg)	RT (°C)	TT (°C)	BG (mg/dl)
Base line	Vehicle	7.377±0.028	39.6±3.1	115.8±20.6	109.2±16.5	37.0±0.2	37.0±0.2	100.6±9.1
	Edaravone	7.385±0.040	35.2±6.0	109.0±16.4	108.4±25.8	37.2±0.1	37.1±0.2	95.0±8.0
1 h after ischemia	Vehicle	7.371±0.026	39.1±4.6	110.4±21.6	105.0±10.9	37.1±0.1	37.1±0.2	90.1±17.0
	Edaravone	7.385±0.043	39.5±5.1	104.4±12.7	107.3±27.6	37.1±0.2	37.0±0.2	80.8±11.0
2 h after ischemia	Vehicle	7.366±0.026	39.2±5.1	112.7±12.3	110.3±12.9	37.2±0.2	37.1±0.2	84.7±18.5
	Edaravone	7.351±0.028	37.0±7.1	108.8±13.7	104.5±24.5	37.1±0.1	37.1±0.2	75.7±10.1
1 h after reperfusion	Vehicle	7.356±0.035	40.3±5.1	107.3±17.6	108.7±19.0	37.1±0.2	37.1±0.2	91.6±15.9
	Edaravone	7.351±0.029	38.5±6.4	104.8±16.1	105.2±27.1	37.2±0.1	37.2±0.1	81.1±11.0

There were no statistically significant differences in any physiological parameters between the groups (unpaired *t*-test). Data are mean±S.D. MABP: mean arterial blood pressure; RT: rectal temperature; TT: temporal muscle temperature; BG: blood glucose level.

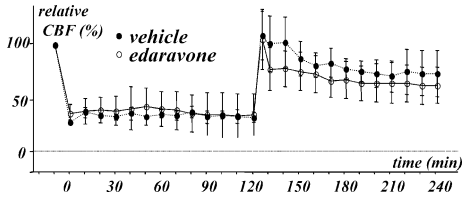

Fig. 1. Cortical cerebral blood flow measured by Laser-Doppler flowmetry in the middle cerebral artery occlusion territory during 2 h of occlusion followed by 2 h of reperfusion. There were no statistically significant differences between the groups at any time points. Values are mean ± S.D.

were significantly increased in the edaravone group compared to those in the vehicle group ($P = 0.030$).

4. Discussion

4.1. Neuroprotective effects of edaravone

The present data revealed that edaravone showed an excellent neuroprotective effect in the cortical area, not in the subcortical area, regardless of cerebral blood flow regulation. Cerebral cortex contains the greater part of the ischemic penumbra, in which cells are so mildly damaged that they can undergo apoptotic pathways rather than necrotic processes in an experimental focal ischemia model (Li et al., 1995). Therefore, cerebral cortex containing a large part of penumbral tissue has been considered as an area that can be pharmacologically rescued even after ischemic event (Hossmann, 1994). Morimoto et al. (1996) reported increased hydroxyl radical formation in the penumbral cortex during middle cerebral artery occlusion using the salicylate hydroxylation method. Kawai et al. (1997) demonstrated that the content of 2-oxo-3-(phenylhydrazono)-butanoic acid, a major oxidation product of edaravone, was elevated in the cortical penumbra using an experimental focal ischemia model. These observations suggest that the neuroprotection by edaravone can be related to preventing cell death mainly in the ischemic penumbra.

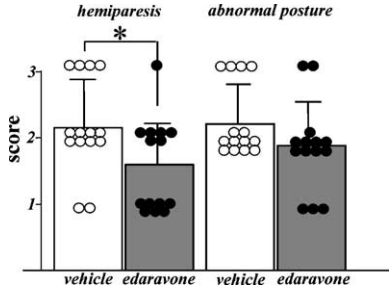

Fig. 2. Neurological scores in the edaravone- and vehicle-treated animals. Hemiparesis scores are 2.1 ± 0.7 in the vehicle-treated group and 1.6 ± 0.6 in the edaravone-treated group with a statistical significance ($P = 0.031$, $n = 14$, each). Abnormal posture scores are 2.3 ± 0.5 in the vehicle-treated group and 1.9 ± 0.6 in the edaravone-treated group without any statistical significance ($n = 14$, each). Values are expressed as mean ± S.D. *$P < 0.05$ by Mann–Whitney U-test.

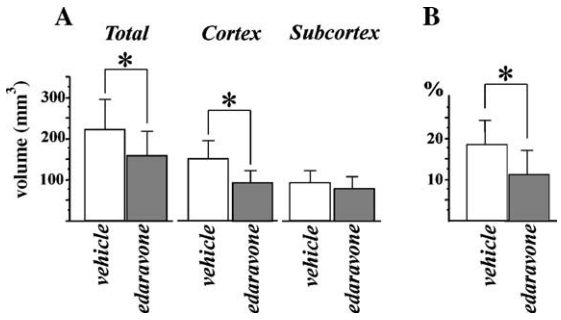

Fig. 3. Infarct volumes and edema index in the edaravone- and vehicle-treated animals. Values are expressed as mean ± S.D. *$P < 0.05$ by unpaired t-test. (A) Infarct volumes: the total infarct volume was significantly reduced in the edaravone-treated group (162.9 ± 59.3 mm^3) compared to that in the vehicle group (227.8 ± 73.7 mm^3). Cortical infarct volume was also significantly smaller in the edaravone-treated group (93.1 ± 36.9 mm^3) compared to that in the vehicle group (142.1 ± 51.6 mm^3). Subcortical infarct volumes were 69.8 ± 34.3 mm^3 in the edaravone-treated group and 85.7 ± 24.2 mm^3 in the vehicle group without statistical difference. (B) Edema index: edema index was significantly improved in the edaravone-treated group (12.1 ± 5.0%) compared to that in the vehicle-treated group (18.1 ± 7.1%).

The present observation also revealed a potent protective effect of edaravone against brain edema. Free radicals derived from arachidonic acid metabolites cause cytogenic deterioration during prolonged cerebral ischemia, and aggravate brain edema formation during reperfusion in transient cerebral ischemia model (Chan et al., 1984; Kogure et al., 1982). These indicated that the toxicity of free radicals, resulting from lipid peroxidation, against brain tissue might be one of the important factors for brain edema formation. Therefore, scavenging free radicals and preventing lipid peroxidation, which are the main

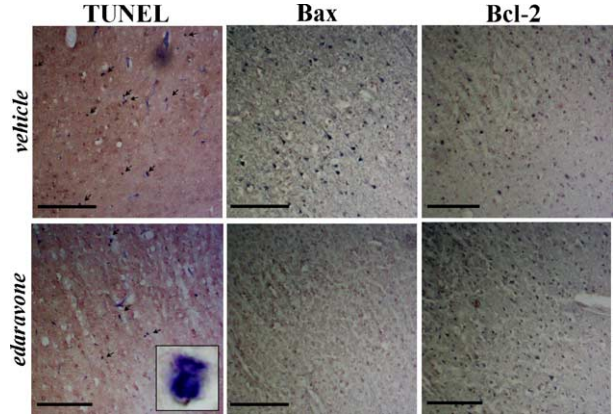

Fig. 4. Representative microphotographs of TUNEL staining and Bax/Bcl-2 immunohistochemistry. TUNEL staining indicates a large number of TUNEL-positive apoptotic cells in the vehicle-treated group compared to the edaravone-treated group. The black arrowheads indicate TUNEL-positive apoptotic cells. Highly magnified microphotograph reveals fragmented TUNEL-positive nuclei, resembling to apoptotic bodies (box). Bax immunohistochemistry shows increased signal in the vehicle-treated group compared to the edaravone-treated group. Bcl-2 immunohistochemistry demonstrates mild immunoreactivity in the vehicle-treated group, while strong up-regulation in the edaravone-treated group. Bars indicate 100 μm.

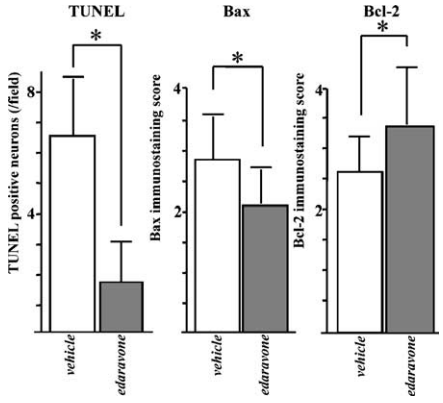

Fig. 5. Semi-quantification for TUNEL staining and immunohistochemistry in the peri-infarct area. The number of TUNEL-positive apoptotic cells significantly reduced in the edaravone-treated group (1.6±1.4) compared to that in the vehicle-treated group (6.4±1.8). Nuclear Bax signal significantly decreased in the edaravone-treated group (2.1±0.8) compared to that in the vehicle-treated group (2.8±0.8), while nuclear Bcl-2 signal significantly up-regulated in the edaravone-treated group (3.4±0.9), compared to the vehicle-treated group (2.7±0.7). Values are expressed as mean±S.D. *$P<0.05$ by Mann–Whitney U-test.

effects of edaravone, can directly suppress brain edema formation.

4.2. Bax/Bcl-2 dependent anti-apoptotic effects of edaravone

The principal finding of this work is that edaravone increased nuclear Bcl-2 expression together with significant decrease in Bax expression in the peri-infarct area. In addition, edaravone significantly reduced the number of TUNEL-positive apoptotic cell in the peri-infarct area, and the Bax/Bcl-2 expression appeared to correlate with the anti-apoptotic effect. The Bcl-2 family proteins are distinct regulators for early stages of apoptosis, and an interaction between pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 may be necessary for the apoptotic process (Merry and Korsmeyer, 1997). Indeed, neurons destined to develop apoptosis reportedly show up-regulation and nuclear translocation of Bax protein parallel to Bcl-2 down-regulation within the same neurons (Isenmann et al., 1998; Krajewski et al., 1995). In addition, Bcl-2 protein is apparent in cortical neurons that survive in the focal ischemia model (Chen et al., 1995), and prevents pro-apoptotic actions of Bax, Bcl-X, or other members of the Bcl-2 family (Merry and Korsmeyer, 1997). Therefore more cells in the peri-infarct area might survive apoptotic cell death in the edaravone-treated animals compared to the vehicle-treated animals.

Recent studies have revealed that antioxidants attenuated ischemic neuronal apoptosis by inhibiting cytochrome *c* release (Kim et al., 2000; Namura et al., 2001). Edaravone has been reported to inhibit both hydroxyl radical generation and iron-induced peroxidative injuries, as well as scavenging superoxide radical (Wu et al., 2000; Shichinohe et al., 2004). Overexpression of SOD-1 reportedly has protective effects against ischemia–reperfusion injury through blocking cytosolic release of cytochrome *c* and reduces apoptosis after transient focal ischemia model (Fujimura et al., 2000). Furthermore edaravone reportedly inhibits the mitochondrial permeability transition pore and up-regulates Bcl-2 expression in a cardiac ischemia model (Rajesh et al., 2003). These observations are consistent with our results that an antioxidant edaravone has neuroprotective effects through the anti-apoptotic effects in the peri-infarct area.

In conclusion, edaravone shows a potent protection against ischemic brain injury in a transient focal ischemia model in rats, and reduces apoptotic cell death through Bcl-2 up-regulation parallel to Bax down-regulation. The present data provide an experimental basis for the clinical evidence that edaravone rescues the ischemic area from deterioration during the acute phase of ischemic stroke.

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