EDARAVONE AND NRF2-INDUCERS AS NEUROPROTECTIVE AGENTS IN HUMAN ASTROCYTES EXPOSED TO HYPOXIA/REOXYGENATION

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Abstract Optimization of neuronal function and survival is an important goal in the treatment of cerebrovascular diseases in order to avoid or improve devastating long-term sequelae. Nerve growth factor (NGF) is essential for neuronal growth and survival in the central nervous system (CNS). Vascular endothelial growth factor (VEGF) is a potent mitogen specific for endothelial cells and a stimulator of neovascularization. VEGF also enhances vascular permeability, which may promote the development of brain edema during cerebral ischemia. These molecules affect the outcome of ischemia/reperfusion injury in the CNS. Edaravone, a brain-penetrant, free radical scavenger, is known to ameliorate postischemic neuronal dysfunction. Transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2), a master regulator of antioxidant responses, plays an important role in the coordinated expressions of stress-inducible genes. Astrocytes express various genes involved in the regulation of neuronal functions, and the regulation of astrocyte gene expressions may be a potential therapeutic target in brain injury. This review aims to appraise the effects of radical scavenger edaravone and a natural Nrf2-inducer as neuroprotective agents in human astrocytes, particularly under an experimental model for hypoxia/reoxygenation.


Key words: Edaravone; Nrf2; Carnosic acid; NGF; Reperfusion

1. Introduction

Thrombolytic therapy with tissue plasminogen activator (tPA) is highly effective for ischemic stroke, by securing reperfusion in the nervous tissue with marginally compromised blood supply. Although the therapeutic time window is currently limited within 3 h after the onset, recent studies suggest that the therapy can be initiated within up to 4.5 h after the onset1,3). However, reperfusion may be associated with a burst of free radicals, which cause oxidative damage to lipids, proteins and nucleic acids. Oxidative damage to mitochondrial membranes triggers the release of cytochrome C and caspase 9, which leads to the activation of caspase 3, the main executioner of cell apoptosis.

Edaravone, a brain-penetrant, free radical scavenger, is known to ameliorate postischemic neuronal dysfunction in patients with acute ischemic stroke4,6). It is expected to be particularly effective in controlling reperfusion injury through

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its antioxidative property. In our previous study, edaravone was also found to regulate the expression of vascular endothelial growth factor (VEGF) in astrocytes\(^7\); and this fact may explain part of deterrent effect of edaravone on brain edema development since VEGF enhances vascular permeability.

One of the major functions of astrocytes is to maintain homeostasis in the central nervous system (CNS) through regulating the coordinated expression of various genes. Thus regulation of astrocyte gene expression may be expected to be a potential therapeutic target in stroke, and this review is intended to address such a therapeutic paradigm employing neuroprotective agents.

2. Edaravone, as a neuroprotective agent

2.1. Development of edaravone

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one (Fig. 1, upper left), MCI-186, RADICUT\(^8\), molecular weight 174.20, Mitsubishi Tanabe Pharma, Osaka, Japan) was developed for the treatment of acute cerebral infarction\(^6,8\), and has been clinically available since June 2001 in Japan\(^8,12\). Edaravone is amphipathic and, thereby, brain-penetrant (permeable to the blood-brain barrier, BBB).

2.2. Edaravone as a free radical scavenger

Edaravone chemically interacts with a variety of reactive oxygen species (ROS) including hydroxyl radical (\(\bullet \)OH)\(^13,14\), peroxy radical (LOO\(^*\))\(^6,13,15\), alkoxyl radical (LO\(^*\))\(^16\), peroxynitrite (ONOO\(^-\))\(^8\) and singlet oxygen (\(\text{O}_2^*\))\(^17\), to which it donates electrons and then is transformed into the stable compound, 2-oxo-3-(phenylhydrazono)-butanoic acid\(^6,8\). Although the high concentration (1 or 3 mmol/L) of edaravone slightly scavenges superoxide anion (\(\text{O}_2^-\))\(^18\), the lower concentration (\(\leq 100 \mu\text{mol/L}\)) of edaravone has no effect on superoxide anion\(^13,19\). Edaravone also quenches hydrogen peroxide (H\(_2\text{O}_2\)) in cellular (neutrophil) and cell-free (xanthin-xanthin oxidase) systems\(^19\). Edaravone suppresses ROS generation and reduces brain edema in a rat ischemic stroke model\(^20\). It also represses delayed neuronal death, induced by ROS, in the hippocampus and cerebral cortex following ischemia in the rat\(^21\).

2.3. Pharmacokinetics of edaravone

2.3.1. Permeability to BBB

Edaravone is demonstrated to have molecular property for its effectiveness in the brain with the ability to permeate to BBB. In an animal study, the ratio of the concentrations of edaravone in plasma and cerebral spinal fluid (CSF) is reported to range from 0.50 to 0.65, indicating that the BBB permeability of edaravone is around 60\%\(^22\).

2.3.2. Metabolism and excretion of edaravone

Plasma concentration of edaravone reaches a maximum level of 17.6 \(\mu\text{mol/L}\) after a single intravenous infusion in a dose of 1.5 mg/kg over 40 min, and after an intravenous infusion of 2.0 mg/kg over 3 h, the concentration reaches a maximum level of 7.0 \(\mu\text{mol/L}\). The plasma concentration reaches a maximum level of 9.3-10.4 \(\mu\text{mol/L}\) after 7 consecutive intravenous infusions, each over 40 min, in a dose of 1.0 mg/kg per day\(^23\). The pharmacokinetics in elderly subjects, over 65 years old, shows a pattern similar to that in younger subjects, indicating a maximum level of 6.0±0.6 \(\mu\text{mol/L}\) after 4 intravenous infusions, each over 30 min, in a dose of 0.5 mg/kg per 12 h\(^24\).

In healthy volunteers (5 men), 87±4\% of edaravone administered by infusion is metabolized and excreted into urine within 24 h\(^25\). Although the urinary excretion of edaravone is slow ranging 0.6-1.0% in 24 h, sulfate or glucuronide conjugation enhances the urinary excretion: 5.6-13.2% and 68.6-83.2% in 24 h, respectively\(^23\).
After a single intravenous administration of $^{14}$C-edaravone (2 mg/kg) to rats, the disappearance of radioactivity from blood vessel is delayed and the radioactivity in blood vessel is detected even after 8 days\(^{25}\). After repeated administration of $^{14}$C-edaravone in a dose of 2 mg/kg/day for 21 days, the radioactivity in blood vessel is detected even after 30 days\(^{26}\). In the brain, no region-specific distribution of edaravone is observed and most of the radioactivity consists of non-metabolized edaravone, whereas most of the radioactivity in the kidney consists of the sulfated form\(^{25}\).

After a single intravenous administration of $^{14}$C-edaravone (2 mg/kg) to male rats, 92.0 ± 1.7% of the radioactivity is excreted into urine within 72 h, whereas 4.6 ±1.2% into bile\(^{25}\). The glucuronide conjugate constitutes 69.6 ±3.1% of the total biliary excretion, and most of it may enter the entero-hepatic circulation and finally excreted from the kidney: 67.3 ±4.5% of the administered dose is excreted into urine\(^{25}\).

3. Edaravone in brain injury
3.1. Effects of edaravone in experimental ischemia and/or reperfusion models

In vitro studies have demonstrated that edaravone inhibits lipid peroxidation and vascular endothelial cell injury\(^{27}\). In rat brain ischemia models, edaravone inhibited brain edema\(^{20,28,29}\), tissue injury\(^{13,30,31}\), inflammatory responses\(^{32}\), lipid free radical formation\(^{33}\) and delayed neuronal death\(^{21}\). In human umbilical vein endothelial cells, edaravone increases endothelial nitric oxide synthase (eNOS) with the inhibition of low-density lipoprotein (LDL) oxidation\(^{34}\). In mice with transient brain ischemia, edaravone suppresses the early accumulation of lipid peroxidation products and oxidative DNA damage, and eliminate a sequence of inflammatory responses resulting in the reduction of inducible NOS\(^{35}\). In rat ischemia/reperfusion models, edaravone is demonstrated to reduce intracellular free Ca\(^{2+}\)-concentration, raise superoxide dismutase (SOD) activity, and decrease mitochondria membrane damage\(^{36}\); and prevents the dissociation of the neurovascular unit (integration of astrocyte endfeet and microvascular basement membrane)\(^{27}\). Edaravone is known to suppress the expression of VEGF in a rat ischemia/reperfusion model\(^{38}\). We confirmed the suppression, by edaravone, of the hypoxia-induced VEGF expression in cultured human astrocytes\(^{7}\).

3.2. Efficacy of edaravone in patients with cerebrovascular diseases

As for the clinical effectiveness of edaravone, several trials have confirmed neuroprotective effects of edaravone in patients with acute cerebral infarction\(^{4,6}\). Several studies demonstrated the efficacy of edaravone in delaying evolution of cerebral infarcts and edema\(^{39}\), reducing the generation of ROS\(^{40}\), oxidized LDL, cytosolic protein S-100B and Mn-SOD\(^{41}\). Improvement of functional outcome in patients with acute ischemic stroke, including lacunar infarction\(^{42}\), cardioembolic stroke\(^{41}\) and aneurysmal subarachnoid hemorrhage\(^{42}\), is also reported\(^{9}\).

3.3. Edaravone in traumatic brain injury

In patients with traumatic brain injury, edaravone is reported to suppress the blood levels of alkoxy radicals that contribute to lipid peroxidation\(^{46}\). Edaravone is also demonstrated to inhibit production of free radicals in a rat model for traumatic brain injury, and this is associated with the protection of neuronal stem cells that have the potential to differentiate into neurons and glia around the injured area\(^{42}\).

3.4. Edaravone in diabetic neuropathy

In the development of diabetic neuropathy, oxidative stress is implicated as a final common pathway. Edaravone is demonstrated to inhibit the augmented angiotensin II-induced contraction in endothelium intact aortic spinal preparations
isolated from thoracic aorta in diabetic rats\textsuperscript{[43]}. Edaravone also shows improving effects on nerve conduction velocity, nociception, lipid peroxidation status, and anti-oxidant enzymes (SOD and catalase) in a rat model of diabetic neuropathy\textsuperscript{[41]}.

### 3.5. Side effects of edaravone

During the phase I study in healthy volunteers, an increase of serum total bilirubin (0.2 mg/kg dose) and a decrease of platelet count (0.2 mg/kg dose) were observed; however, they were recovered 1 w after the infusion. No other problems were observed, and edaravone was regarded to have a good tolerability in healthy volunteers\textsuperscript{[25]}.

Adverse reactions to edaravone, including renal and hepatic disorders, have been reported after its launch in June 2001, and renal disorders have been the most frequent and sometimes serious. In a study reviewed 207 Japanese patients who were treated with edaravone for acute stroke and developed renal disorder, no particular factor other than edaravone administration was found as a possible cause for the renal disorders in 17 patients (8.2%), whereas factors other than edaravone were associated with renal disorders in the remaining 190 patients\textsuperscript{[45]}. The frequency of renal dysfunction as a complication of edaravone treatment is estimated to be 0.04% (207/530000) since edaravone was given to 530000 patients in Japan during the same period of analysis, June 2001 to September 2005\textsuperscript{[45]}.

In the 207 patients with renal disorders, the overall recovery rate of renal function was 43%; risk factors for the nonrecovery of renal function were the complication of severe infection and the implication of blood purification, and risk factors for death were advanced age (> 80 years) and the complication of severe infection\textsuperscript{[46]}.

The precise role(s) of edaravone in the pathogenesis of renal disorders remain(s) unclear. Early detection of renal disorders and control of infection are critical during edaravone treatment. Also, it has been pointed out that care should be taken with the clinical use of edaravone when pterin derivatives stay in the body\textsuperscript{[47]}.

### 4. Therapeutic potential of Nrf2-inducers

Carnosic acid (Fig. 1, upper right), a component of rosemary (Rosmarinus officinalis L.), induces a transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2), a master regulator of antioxidant response, by inhibiting Keap1 (Kelch-like ECH-associated protein 1)\textsuperscript{[48-50]}. Keap1 is a bifunctional protein that serves as an Nrf2-specific adaptor for the Cul3 ubiquitin ligase complex\textsuperscript{[51,52]} and a sensor for oxidative and/or electrophilic stresses\textsuperscript{[53]}. Keap1 has many reactive cysteine residues that have the potential to sense various electrophiles\textsuperscript{[54]}. Nrf2 plays an important role in the coordinated expression of many phase 2 detoxifying enzymes such as glutathione S-transferases, heme oxygenase-1 and NAD(P)H quinone oxidoreductase\textsuperscript{[55,56]} and is considered as an indicator and modulator of oxidative stress in neurodegeneration\textsuperscript{[57]}. Nrf2 expression in astrocytes, by overexpression, prevents neuronal death in a mouse model of Parkinson’s disease\textsuperscript{[58]}.

The Nrf2 pathway is also activated by other electrophilic compounds including natural products such as sulforaphane\textsuperscript{[59]} and curcumin\textsuperscript{[60]} (Fig. 1, bottom left and right). The mitogen-activated protein kinases (MAPK) are activated by phase 2 gene inducers and involved in survival response leading to the transcriptional activation of defense genes mediated by ARE/EpRE (antioxidant or electrophile response element)\textsuperscript{[61]}. A marked nuclear accumulation of Nrf2 protein is reported in cultures of HK-2 human renal proximal tubular epithelial cells or T84 human intestinal epithelial cells exposed to hypoxia\textsuperscript{[62]}. Some Nrf2-inducers may be a potential modulator of the expression of
neuroprotective genes in astrocytes and useful as therapeutic agents.

5. Astrocytes as a therapeutic target in ischemic brain injury

5.1. Regulation of VEGF in astrocytes

Astrocytes play integral roles in maintaining homeostasis and regulating responses to various stresses in the CNS\(^{63,64}\). Hypoxia upregulates many genes in astrocytes, including glycolytic enzymes and angiogenic growth factors as \(VEGF^{65,66}\). VEGF, a potent mitogen specific for endothelial cells and a stimulator of neovascularization, is also known to enhance vascular permeability\(^{67,70}\), which may be involved in the development of brain edema during cerebral ischemia\(^{71}\). A neutralizing antibody against VEGF is reported to reduce infarct volume in a rat 2-vein occlusion model\(^{72}\). Edaravone may protect ischemic brain tissue from the development of vasogenic edema, in part, through the suppression of the enhanced \(VEGF\) transcription in astrocytes\(^{73}\).

5.2. Regulation of nerve growth factor (NGF) in astrocytes

Neurotrophins including NGF are essential for neuronal growth and subsequent survival\(^{73}\). Astrocytes continue to produce NGF\(^{74,76}\), and its circulating and brain levels undergo significant variations after exposure to stressful events\(^{77}\).

For instance, transient focal cerebral ischemia enhances the expression of NGF in reactive astrocytes particularly in the peri-infarct penumbra\(^{78}\). In our previous study, platelet-activating factor (PAF), a proinflammatory phospholipid\(^{79}\), was found to enhance the NGF expression in human astrocytes\(^{80}\). Since PAF is generated in high levels in ischemic brain tissue\(^{81,82}\), it may play a dual role, by promoting inflammation and protecting neuronal cells, in ischemic brain injury. Also, carnosic acid is demonstrated to upregulate the NGF expression in T98G human glioblastoma cells\(^{83}\). NGF exerts protective effects on cultured neurons against a variety of deleterious factors\(^{84,86}\).

Signaling pathways through MAPK\(^{87,88}\) (including extracellular signal-regulated kinase (ERK)\(^{89}\)) and phosphatidylinositol 3-kinase (PI3K)\(^{90}\) are known to mediate NGF expression in astrocytes. Ethanol extracts of an edible mushroom, \(Hericium erinaceus\) (Yamabushitake), are reported to promote NGF expressions for mRNA and protein, via c-Jun N-terminal kinase (JNK) signaling, in 1321N1 human astrocytoma cells\(^{91}\). Also, JNK pathway is likely to mediate the astrocyte NGF expression in response to edaravone\(^{92}\) or carnosic acid (Yoshida et al., unpublished data).

5.3. Perspectives

The benefit of edaravone as a therapeutic measure against ischemic stroke is almost established both from the viewpoints of immediate prognostic assessment and from functional outcome of the patients in chronic stage. Thrombolysis with tPA is now an essential therapeutic strategy in the treatment of acute cerebral infarction, and edaravone is suggested to be useful for the extension of the therapeutic time window for thrombolytic therapy\(^{6,35,93}\). The combination of neuroprotective agents, such as edaravone or carnosic acid, with tPA may be considered as a current standard
in the treatment of acute cerebral infarction; and this is expected to expand the indication of thrombolytic therapy beyond 3 h after stroke.

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