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Edaravone directly reacts with singlet oxygen and protects cells from attack

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ABSTRACT

Aims: Protective effects of edaravone, an approved medicine for acute brain infarction in Japan, on cell death induced by singlet oxygen ($^1O_2$) were examined.
Main method: The $^1O_2$ scavenging activity was examined by direct analysis of near-infrared luminescence in a cell-free system and by fluorospectrometry in the presence of cells. The protective effects of edaravone on $^1O_2$-induced cell death were examined, using rat neuronal B50 cells. Cell death was evaluated by mitochondrial respiration (MTT assay), confocal microscopy and time-lapse imaging. The chemical reaction of edaravone with $^1O_2$ was examined by production analysis using high performance liquid chromatography (HPLC).
Key findings: When rose Bengal (RB) in D_2O was irradiated by a 514 nm laser beam, the signal of $^1O_2$ was observed. Edaravone suppressed the $^1O_2$ signal more potently than azide, a $^1O_2$ scavenger. When B50 cells were irradiated by 525 nm green light in the RB solution, production of $^1O_2$ and induction of cell death were observed. The fluorospectrometric study and the MTT assay revealed that 100–400 μM edaravone suppressed the $^1O_2$ production and attenuated cell death in a concentration-dependent manner. Confocal microscopy and the time-lapse imaging revealed that edaravone prevented the impairment of membrane integrity and the progression of cell death induced by $^1O_2$. The HPLC study revealed that edaravone chemically reacted with $^1O_2$ and changed another compound.
Significance: Since $^1O_2$ is possibly involved in post-ischemic neuronal damage, the clinically approved curative effects of edaravone on acute brain infarction might be attributed to its potent $^1O_2$ scavenging activity.

Introduction

There is a growing body of evidence that edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a free radical scavenger, has neuroprotective effects (Watanabe et al. 1994; Kawai et al. 1997; Zhang et al. 2005; Wu et al. 2006), and it has been widely used in patients with acute brain infarction in Japan (Group TEABIS 2003). Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) are suggested to play important roles in neuronal cell death (Ishikawa et al. 1999). Recently, in addition to these ROS, singlet oxygen ($^1O_2$) has become known as an effector of neuronal damage (Valencia and Moran 2004). Among these ROS, edaravone is known to scavenge •OH and H_2O_2 (Yamamoto et al. 1997; Mikawa et al. 2005). Low reactivity of edaravone to $O_2^{\bullet-}$ has also been reported (Watanabe et al. 1994; Mikawa et al. 2005). In the previous study, we reported that edaravone suppressed the release of $^1O_2$ from activated neutrophils with an IC_{50} of approximately 0.3 μM, which indicated that edaravone was a potent $^1O_2$ scavenger (Sommani et al. 2007). However, the inhibition of $^1O_2$ release from activated neutrophils does not necessarily imply the direct scavenging of $^1O_2$. Activated neutrophils first generate $O_2^{\bullet-}$ by NADPH-oxidase activity, and generated $O_2^{\bullet-}$ is dismuted to H_2O_2. H_2O_2 reacts with hypochlorite (HOCl) produced by myeloperoxidase (MPO) activity to form $^1O_2$ (Klebanoff 2005). Therefore, the inhibitory effect of edaravone on $^1O_2$ release might be attributed to that on $O_2^{\bullet-}$ production by NADPH-oxidase activity. Otherwise, edaravone might inhibit the $^1O_2$ formation by MPO activity. Further evidence is required to determine if edaravone directly scavenges $^1O_2$. More importantly, assuming that edaravone has $^1O_2$ scavenging activity, the protective effects of edaravone on the $^1O_2$-induced cell death need to be evaluated, because there is no report of edaravone as a curative agent for $^1O_2$ insult.

In the present study, first to elucidate whether edaravone directly scavenges $^1O_2$, the scavenging activity of edaravone against photochemically generated $^1O_2$ was examined. In a cell-free system, the $^1O_2$

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scavenging activity of edaravone was examined by direct analysis of near-infrared luminescence at 1270 nm, and the scavenging activity was compared with azide, a $^1O_2$ scavenger. In the presence of rat neuronal B50 cells, the $^1O_2$ scavenging activity of edaravone was examined by fluorospectrometry, using a $^1O_2$ specific probe, singlet oxygen sensor green® (SOSG). Then, the protective effects of edaravone on $^1O_2$-induced cell death of B50 cells were evaluated by mitochondrial respiration (MTT assay), fluorescence confocal microscopy and time-lapse cell imaging. Finally, the chemical reaction of edaravone with $^1O_2$ was examined by production analysis using high performance liquid chromatography (HPLC).

Materials and methods

Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal calf serum (FCS), penicillin–streptomycin liquid and Hanks' balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA); deuterium oxide (D_2O) was from Wako Pure Chemicals (Osaka, Japan); sodium azide was from Nacalai Tesque (Kyoto, Japan); rose Bengal (RB) was from Sigma Chemicals (St. Louis, MO); and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Dojin Chemical (Kumamoto, Japan). Singlet oxygen sensor green® (SOSG) and LIVE/DEAD® cell vitality assay kit with C_{12}-resazurin and Sytox Green® were purchased from Molecular Probes (Eugene, OR). Edaravone was a kind gift from Tanabe-Mitsubishi Pharma Corporation (Tokyo, Japan). Edaravone was directly dissolved in HBSS or D_2O to a final concentration of 4 mM, pH 7.4, and filtered for sterilization.

Detection of $^1O_2$ by direct analysis of near-infrared luminescence

Scavenging activity of edaravone against $^1O_2$ in a cell-free system was examined by direct analysis of near-infrared luminescence using a Raman spectroscopy (LabRAM HR-800, HORIBA, Kyoto, Japan). $^1O_2$ was photochemically generated by irradiation of RB solutions. Because of the short lifetime of $^1O_2$ in H_2O (3.8 μs), D_2O was used as the solvent in all experiments, where the lifetime is much longer (62 μs). The quantum yields of $^1O_2$ production were determined by measurements of the $^1O_2$ luminescence at 1270 nm, which was produced during continuous irradiation of 10 μM RB solutions by a laser beam (wavelength 514.5 nm, power 3 mW). The luminescence of $^1O_2$ was measured with a liquid nitrogen-cooled InGaAs photodiode in conjunction with a 1270 nm interference filter.

Cell culture

Rat neuronal cell line B50 was obtained from the European Collection of Cell Cultures (Wiltshire, UK) and cultured in RPMI medium containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator.

Detection of $^1O_2$ using a $^1O_2$-specific probe

Scavenging activity of edaravone against $^1O_2$ in the presence of cells was examined by fluorospectrometry using a $^1O_2$-specific probe, singlet oxygen sensor green® (SOSG). B50 cells (2×10^5 cells/ml) were incubated overnight in 12-well tissue culture plates (BD Biosciences, Bedford, MA) with 1 ml culture medium in each well. The medium was replaced with HBSS alone or containing 2 μM RB in the presence or absence of 100–400 μM edaravone or 4 mM azide and irradiated for 5 min with an experimental lamp ($\lambda_{max}=525$ nm; 72 W/m^2; CCS Inc., Kyoto, Japan). HBSS was used to avoid light-dependent ROS generation in cell culture media (Grzelak et al. 2001). The $^1O_2$ production was determined by measuring the fluorescence of SOSG using a fluorometric microplate reader (Fluoroskan Ascent, Labsystems, Helsinki, Finland) with excitation and emission wavelength of 485 and 504 nm, respectively.

MTT assay

Effects of edaravone on $^1O_2$-induced cell death were quantitatively measured by mitochondrial respiration (MTT assay). B50 cells (2×10^5 cells/ml) were incubated overnight in 12-well tissue culture plates with 1 ml culture medium in each well. The medium was replaced with HBSS alone or containing 200 nM RB in the presence or absence of 100–400 μM edaravone or 4 mM sodium azide and irradiated for 5 min with an experimental lamp ($\lambda_{max}=525$ nm). Cell viability after irradiation was assessed by the mitochondrial-dependent reduction of MTT to formazan. The extent of reduction of MTT to formazan within cells was quantified by measurement of the absorbance at 550 nm.

Fluorescence confocal microscopy

Effects of edaravone on $^1O_2$-induced cell death were visually evaluated by fluorescence confocal microscopy. B50 cells (4×10^5 cells/ml) were incubated overnight in CELLview GlassBottom CellcultureDish® (Greiner Bio-One GmbH, Frickenhausen, Germany). This dish is divided into 4 fractions by septa and each fraction has a capacity of 500 μl. The medium was replaced with HBSS containing 200 nM RB in the presence or absence of 400 μM edaravone or 4 mM sodium azide and irradiated for 5 min with an experimental lamp ($\lambda_{max}=525$ nm; CCS Inc., Kyoto, Japan). After irradiation, cells were stained with 2 μM C_{12}-resazurin, a metabolic marker, and 50 nM Sytox Green, a vital dye. Cell vitality was visualized using a laser-scanning confocal microscope (Nikon Digital Eclipse C1, Nikon, Tokyo, Japan); for this, the images of the cells stained with the two dyes were obtained under 488 nm radiation for the green fluorescence of Sytox Green and under 543 nm radiation for the red fluorescence of C_{12}-resazurin, followed by the electronic merging of images.

Time-lapse cell imaging

Effects of edaravone on$^1O_2$-induced cell death was sequentially observed using a time-lapse imaging system (BioStation IM, Nikon), which consists of an incubator incorporating fluorescent microscopy equipped with a digital CCD camera and a PC computer for data acquisition and analysis. Cells (4×10^5 cells/ml) were incubated overnight in the above-mentioned CELLview GlassBottom CellcultureDish®. The medium was replaced with HBSS containing 1% FCS, 200 nM RB and Sytox Green (final 100 nM), a vital dye. Edaravone (400 μM) was added to one fraction. The dish was mounted on the time-lapse imaging system. Inside the system, cells were incubated at 37 °C and 5% CO_2, and phase contrast images and fluorescent images were acquired every 5 min for 4 h. Green fluorescence images for Sytox Green were acquired using a GFP filter (Nikon). $^1O_2$ was generated by photoactivation of RB with green light for 8 s every 5 min using a TRITC filter (Nikon) and a mercury lamp equipped with the microscope.

HPLC study

To determine the product from the reaction of edaravone with RB under 525 nm green light radiation, production analysis was performed using high performance liquid chromatography (HPLC). The HPLC system consisted of a Waters 2695 Separations Module (Waters, Milford, MA) equipped with a vacuum degasser and a temperature controlled column compartment. UV detection was done with a Waters model 996 photodiode-array detector (Waters). The detector was set at 210–400 nm. For HPLC, a COSMOSIL 5C18-MSII octadecylsilane column (4.6×150 mm and particle size 5 μm; Nacalai Tesque; Kyoto, Japan) was used. Isocratic elution with ammonium