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PROTECTIVE EFFECTS OF EBSELEN, A SELENO-ORGANIC ANTIOXIDANT ON NEURODEGENERATION INDUCED BY HYPOXIA AND REPERFUSION IN STROKE-PRONE SPONTANEOUSLY HYPERTENSIVE RAT

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Abstract—Cerebral ischemia followed by oxygen reperfusion induced apoptosis in hippocampal neurons in the stroke-prone spontaneously hypertensive rat (SHRSP) but not in Wistar Kyoto rats (WKY). We investigated whether 2-phenyl-1,2-benzisoselenazol-3(2H)-one, also called PZ51 (ebselen), useful for treating ischemic damage or antihypertension in the brain, can protect against ischemic neuronal damage in SHRSP. In this study, we compared the effects of ebselen, carvedilol, 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186) as well as vitamin E, added to cultures of neurons after reoxygenation (20% O_2) following hypoxia (1% O_2). SHRSP neurons died rapidly during reoxygenation following hypoxia but were rescued in large measure by 10 μM ebselen (neuronal death; 2.7±1.4%). In order of neuroprotective potency, the agents ranked as follows: ebselen>carvedilol>MCI-186>vitamin E. *In vivo*, strong neuroprotection by ebselen was observed in the hippocampal CA1 region of SHRSP (32.9±9.5 apoptotic neuron/1000 neurons, 30 mg/kg/day). Ebselen prevented apoptosis as confirmed by morphological observations *in vivo*. Its effect was associated with the expression of Bcl-2 and Bax. These findings suggest that ebselen has a marked inhibitory effect on neuronal damage during stroke. Ebselen may be effective in the prevention and/or treatment of neurodegenerative diseases associated with excessive apoptosis in patients with stroke.

Key words: apoptosis, ebselen, ischemic neuronal death, hypoxia, SHRSP.

The stroke-prone spontaneously hypertensive rat (SHRSP) is an experimental model of malignant hypertension and has a high incidence (95%) of cerebrovascular disease (Okamoto et al., 1974; Yamori et al., 1974). An increase in sodium intake accelerates the rise in blood pressure and the appearance of cerebral vasogenic edema in SHRSP (Nagaoka et al., 1976). Cerebral ischemia for 20 min in SHRSP induced a massive efflux of glutamate, causing delayed neuronal death in region CA1 of the hippocampus, whereas the parental strain of SHRSP, Wistar Kyoto rats (WKY), lacked these characteristics under the same conditions (Gemba et al., 1992). SHRSP, which produce more hydroxyl radicals than WKY, are susceptible to neuronal damage (Negishi et al., 2001). We demonstrated that cerebral ischemia followed by reperfusion significantly increased the number of apoptotic neurons in SHRSP, although pretreatment with vitamin E reduced the apoptotic cell count (Tagami et al., 1998, 1999a,b). In addition, we demonstrated that the mechanism of neuronal injury in SHRSP is related to the vulnerability of neurons to oxidative stress (Yamagata et al., 2000). More importantly, we pointed out that the antihypertensives amlodipine and carvedilol prevent cytotoxicity in cortical neurons in SHRSP during hypoxia and reoxygenation (H/R) (Yamagata et al., 2004). These reports suggest that antioxidative properties along with antihypertensive activity inhibit neuronal cell death in the treatment of cerebrovascular stroke and neurodegenerative diseases in hypertensive patients.

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one, also called PZ51), a seleno-organic compound known to have anti-oxidative and anti-inflammatory effects, prevents ischemia-induced cell death (Martinez-Vila and Sieira, 2001). Its putative mode of action as a neuroprotectant is via cyclical reactions of reduction and oxidation (Imai et al., 2001), in a manner akin to glutathione peroxidase (Sies, 1993; Ren et al., 2001). Recently, it was demonstrated that ebselen is a substrate for thioredoxin reductase, strongly stimulating its hydroperoxide reductase activity, and a rapid thioredoxin oxidant in mammals (Zhao and Holmgren, 2002). Moreover, it has been reported that ebselen decreased oxidative stress-induced apoptosis through the inhibition of the c-Jun N-terminal kinase (JNK) and activator protein-1 (AP-1) signaling pathway but not extracellular signal-regulated kinase (ERK)1/2 (Yoshizumi et al., 2002). The anti-ischemic agent MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5-one) also called edaravone is a potent scavenger of hydroxyl radicals that also inhibits iron-induced peroxidative injuries (Watanabe et al., 1988). Reports have demonstrated that MCI-186 has protective

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)-one, also called PZ51; FBS, fetal bovine serum; H/R, hypoxia and reoxygenation; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; MCA, middle cerebral artery; MCI-186, 3-methyl-1-phenyl-2-pyrazolin-5-one; PBS, phosphate-buffered saline; SHRSP, stroke-prone spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

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effects on both transient cerebral ischemia and hemispheric embolization in rats (Watanabe et al., 1994; Imai et al., 2003). Moreover, MCI-186 significantly decreased the size of cerebral infarcts after occlusion of the middle cerebral artery (MCA) (Kawai et al., 1997).

Although it has been reported that ebselen and MCI-186 are effective for treating oxidative stress–induced neuronal damage both *in vivo* and clinically, the precise actions of these agents in SHRSP neurons have not yet been elucidated (Kawai et al., 1997; Yuki and Kogure, 1997; Imai et al., 2001; Ikeda et al., 2002). Therefore, we were interested in whether there is a neuroprotective effect on ischemic-induced neuronal damage in SHRSP caused by ebselen or MCI-186. The purpose of the present study was to induce cell death through H/R in cultured cortical neurons isolated from WKY and SHRSP and examine whether ebselen can protect against ischemic neuronal damage in region CA1 of the SHRSP hippocampus *in vivo*.

EXPERIMENTAL PROCEDURES

Neuronal culture

We cultured neurons from the fetal brain which were not influenced by blood pressure. Namely, primary dissociated neurons were prepared from fetal WKY and SHRSP (15 days of gestation) as described previously (Tagami et al., 1997, 1998; Yamagata et al., 2004). The brains from fetal rats were dissected, and all blood vessels and pia mater were removed. The brains were minced into 2–3 mm pieces, which were then washed in phosphate-buffered saline (PBS) and placed in PBS containing 200 μg/ml of streptomycin and 1 mg/ml of ceftazidime. The brain pieces were treated with 0.25% trypsin at 37 °C for 10 min and digested with 0.15 U/ml of papain (Funakoshi, Tokyo, Japan), 0.02% L-cysteine monohydrochloride (Sigma Chemical Co., Tokyo, Japan), 0.02% bovine serum albumin (Sigma) and 0.5% glucose in PBS at 37 °C for 10 min. The dissociated cells were collected by centrifugation and resuspended in Dulbecco's modified Eagle's medium (DMEM; Nissui Phamaceutical, Tokyo, Japan). After a gentle mechanical trituration through a siliconized Pasteur pipette, cells were filtered through a sterile lens paper filter. The cells in the filtrate were collected by centrifugation, resuspended in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco-BRL Life Technologies, Tokyo, Japan), and then plated on a poly-L-lysine-coated 24-well culture plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at a density of $3–4\times10^5$ cells per well. This point in time was designated as day 0. Forty-eight hours after plating, the cells were treated with 40 μg/ml of 5-fluoro-2′-deoxyuridine (Sigma) to prevent the proliferation of non-neuronal cells. The cells were maintained in DMEM with 5% FBS+5% horse serum (Gibco-BRL Life Technologies) in a humidified 5% CO_2 atmosphere at 37 °C. The medium was changed every 2 days. The study was started when nerve fibers and dendrites were well developed (days 5–6). In the experiments, culture conditions such as the number of cells were the same. Therefore, differences between the strains are unlikely to be due to culture conditions or individual differences in the same strain.

Treatment of cultures

Ebselen and carvedilol were provided by Daiichi Pharmaceutical Corporation, Ltd. (Tokyo, Japan). MCI-186 was provided by Mitsubishi Chemical Corporation (Tokyo, Japan). Vitamin E (alpha-D-L-tocopherol) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ebselen and carvedilol were dissolved in fresh dimethylsulfoxide (DMSO, Sigma) before their addition to the aqueous solvents. Concentrations of DMSO were less than 0.5% of the solvent buffer and were shown to dissolve the drug effectively. MCI-186 was dissolved in ethanol and diluted with a culture medium to give a final ethanol concentration below 0.01%. Vitamin E was dissolved in DMEM supplemented with 5% FBS+5% horse serum with the final concentration of ethanol being less than 0.01%.

When neurons in culture became mature (days 5–6), they were incubated in 1% O_2, 94% N_2, and 5% CO_2 (hypoxia) for 24 h. After this period of hypoxia, they were maintained in air (about 20% O_2) and 5% CO_2 for 3 h (reoxygenation). In addition, mature neurons were cultured in air (about 20% O_2) and 5% CO_2 for 6–48 h to serve as a control. Immediately, after ebselen, MCI-186, vitamin E or carvedilol was added to the neuronal cells, the oxygen concentration was reduced and the cells were maintained in 1% O_2, 94% N_2, and 5% CO_2, in a humidified atmosphere at 37 °C for 24 h (hypoxia). When the hypoxic culture was finished, the agent was again added and the cells were incubated in air (about 20% O_2) and 5% CO_2, in a humidified atmosphere at 37 °C for 3 h.

Evaluation of neuronal cell death

Viable and non-viable cells were counted under an electron microscope by two of the authors as reported (Tagami et al., 1998, 1999a; Yamagata et al., 2004). Morphological observation revealing a large quantity of lipid droplets in the cytoplasm and cytoplasmic electron density was used to define a dead cell (Tagami et al., 1999a). That is, cell death was the number of morphologically identified dead cells compared with the total number of neuronal cells. Furthermore, cells were determined as intact, necrotic, or apoptotic based on criteria described previously (Tagami et al., 1998). The criteria are as follows: (l) cells lose their axons and dendrites and numerous lipid droplets appear in the cell bodies, although cell organelles remain intact (initial stage of apoptosis); (2) cells become round, small and electron-dense, and nuclei demonstrate prominent invaginations (second stage of apoptosis); (3) cells lose their cytoplasm and cell membrane, and nuclei become small and dark, then disappear (advanced stage of apoptosis); (4) cells become electron-lucent, the organelles decrease in number, and the nuclei demonstrate abnormal clusters of chromatin (primary or secondary necrosis).

Lactate dehydrogenase (LDH) activity

When the experiments were finished, we measured the LDH activity released from the damaged cells in a bathing medium as reported previously (Tagami et al., 1998). The data represent the mean of 12 cultures pooled from three experiments. We designated the mean LDH activity of SHRSP neurons (10^6 cells/ml) just before the hypoxic culture as 100%.

Electron microscopic examination of cultured neuronal cells

The cultures were terminated by fixing the cells with 1.25% glutaraldehyde (Wako) and 1% paraformaldehyde (Wako) in 0.1 M PBS for 30 min. The cells were washed overnight at 4 °C in the same buffer, and post-fixed with 2% OsO_4 (Wako) buffered with 0.1 M PBS for 1 h. The cells were dehydrated in a graded series of ethanol concentrations and embedded in Epon 812. Ultrathin sections were double-stained with uranyl acetate and lead citrate for electron microscopic examinations. We performed at least three experiments per cultivating condition and examined at least three wells (diameter 10 mm) in each experiment. We examined over 3000 neurons in each well. Consequently, we obtained data on 10,000 neurons per condition (Tagami et al., 1998).

Experiments with SHRSP *in vivo*

We used 10 male WKY (8 weeks, blood pressure 131±3 mm Hg, body weight 210–230 g) and 10 male SHRSP (8 weeks, 180±6