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Idebenone protects hippocampal neurons against amyloid β-peptide-induced neurotoxicity in rat primary cultures

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Abstract The application of amyloid β-peptide (Aβ) 1–40 (10 μM) caused neurodegeneration of hippocampal neuronal cells, as indicated by the release of lactate dehydrogenase (LDH) into the culture medium. Treatment with idebenone (10–1000 nM), a potent antioxidant in mitochondria, protected the hippocampal neurons against the Aβ1–40 (10 μM)-induced neurotoxicity. To determine the morphological change in neurons during the Aβ1–40-induced cytotoxicity, the cells were immunostained with anti-MAP2 antibodies. After 4-day exposure to 10 μM Aβ1–40, the number of neurons was reduced, and the surviving neurons had an apparently reduced number of neurites which were shorter than those of control neurons. When idebenone was added to the culture medium with Aβ1–40, the number of surviving neurons was significantly increased, and their neurites were as long as seen in control culture. These results suggest that reactive oxygen species mediate neurotoxicity of Aβ1–40, and idebenone protects neurons against the Aβ1–40-induced neurotoxicity.

Key words Amyloid β-peptide · Idebenone · Neurotoxicity · Oxidative stress · Alzheimer's disease · Hippocampal neurons

Introduction

Senile plaques, a neuropathological feature of Alzheimer's disease (AD), consist primarily of an insoluble aggregate of amyloid β-peptide (Aβ), a 40- to 43-amino acid peptide (Glenner and Wong 1984; Masters et al. 1985). Dense core plaques of Aβ deposited in the brain of AD patients are typically surrounded by dystrophic neurites and glial cells (Selkoe 1991). The deposition of amyloid fibrils in senile plaques appears to be dependent on the rate of insoluble complex formation from soluble protein as well as on the rates of production and removal of Aβ. Cell culture studies have shown that Aβ can be directly neurotoxic (reviewed by Yankner 1996) and furthermore aggregation of Aβ is essential for its neurotoxicity (Pike et al. 1993). Recently, it has been shown that reactive oxygen species (ROS) promote aggregation of Aβ and enhance its neurotoxicity (Dyrks et al. 1992; Pike et al. 1993; Smith et al. 1995). Moreover, Aβ generates ROS (Hensley et al. 1994) as demonstrated by increased levels of H_2O_2 and lipid peroxides in cultured cells (Behl et al. 1994; Pike et al. 1997), which can damage and kill neurons (Miyamoto et al. 1989; Murphy et al. 1989). Thus, both H_2O_2 and related peroxides can be implicated in Aβ-induced neurotoxicity.

Glutamate causes delayed and concentration-dependent cytotoxicity in the N18-RE-105 neuronal cell line, in which glutamate causes an inhibition of cystine uptake into the cells and a marked decrease in cellular glutathione (GSH) synthesis which results in exposure of the cells to oxidative stress (Murphy et al. 1989). Idebenone, 6-(10-hydroxydecyl)-2,3-dimethoxy-1,4-benzoquinone, has been shown to protect the cells from glutamate (10 mM)-induced cytotoxicity in a concentration-dependent manner without affecting the reduction in cellular GSH levels (Miyamoto et al. 1989). Another in vivo study evidenced that repeated administration of idebenone protects striatal neurons against the neurotoxicity induced by the intrastriatal injection of excitotoxins in rats (Miyamoto and Coyle 1990). Neuronal cell death caused by excitatory amino acids is also thought to be promoted by ROS (Coyle and Puttfarcken 1993). These findings indicate that idebenone rescues the cells that are exposed to oxidative stress from cytotoxicity. Here, we examined the effects of idebenone on the Aβ-induced neurotoxicity in hippocampal neuronal cultures.

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Materials and methods

Primary culture and drug treatment. Hippocampi of embryonic (18-day) rats (Sprague-Dawley; Charles-River Laboratories, Yokohama, Japan) were used for the preparation of dissociated neuronal cell cultures. Brains were rapidly removed, and the hippocampi were dissected free in ice-cold Hanks' balanced salt solution (HBSS). Hippocampi were suspended in HBSS containing 0.25% trypsin and 0.01% deoxyribonuclease I, and incubated for 10 min at 37°C. The cells were plated on poly-L-lysine coated 48-well plates (Costar, Cambridge, Mass., USA) and grown for 3 days in minimum essential medium (MEM) containing 10% fetal bovine serum, 1% B27 supplement (×50; Life Technologies, Rockville, Md., USA) and 22 mM D-glucose. After culturing for 3 days, the medium was changed to MEM containing 10 mM HEPES-Na (pH 7.3), N2 supplement and 22 mM D-glucose. On the seventh day of the culture, the cells were exposed to 10 μM Aβ1–40 (Lot No. 510597; Bachem, Bubendort, Switzerland) with or without idebenone. Aβ1–40 was dissolved in Ca^{2+}, Mg^{2+}-free phosphate buffered saline (PBS) and incubated at 37°C for 5 days before being added to the culture medium. Three or 4 days after exposure to Aβ, neuronal cells were observed morphologically with a phase-contrast microscope and lactate dehydrogenase (LDH) released from damaged cells into the medium was measured spectrometrically by using a diagnostic kit (Wako, Osaka, Japan).

MAP2 staining. Cultured cells were fixed for 30 min in 4% paraformaldehyde in PBS at room temperature. After pre-incubation with PBS containing 5% (v/v) normal goat serum and 0.3% (v/v) Triton X-100 twice for 5 min at room temperature, the cells were incubated with anti-microtuble-associated protein 2 (MAP2) antibody (1:5,000; Amersham, Buckinghamshire, UK) overnight at 4°C. Immunostaining was then carried out with a Vectastain ABC kit (Vector, Burlingame, Calif., USA). Visualization was carried out with a peroxidase staining kit (Wako, Osaka, Japan). Stained neurons with intact neurites of uniform diameter and a soma with a smooth round appearance were counted as viable neurons in the field of photograph (=3.91 mm^2).

Statistical analysis. For statistical analysis, a one-way or two-way analysis of variance (ANOVA) was used, and Dunnett's test was used post-hoc.

Results

Addition of Aβ1–40 (10 μM) caused neurotoxicity in rat hippocampal neuronal culture, as demonstrated by a significant increase in the release of LDH into the medium and reduction of the number of neuronal cells. The effects of idebenone on the Aβ1–40-induced neurotoxicity in rat hippocampal neurons are shown in Figs. 1 and 2. Idebenone alone had no significant effects on the amount of LDH released from the cells or the number of cells measured 4 and 5 days after application, respectively (Fig. 1, 2). Even in control culture, there was some background LDH release from the cells, but this was not changed by treatment with idebenone, suggesting there was no significant oxidative damage in the control culture. However, idebenone showed significant effects on the increase in LDH release and the reduction of the number of neuronal cells caused by Aβ1–40. A one-way ANOVA on LDH release revealed a significant group effect [F(3,8)=50.5, $P<0.01$]. Follow-up comparison using Dunnett's test showed that treatment with idebenone (10–1000 nM) significantly inhibited the release of LDH into medium at all concentrations tested ($P<0.01$; Fig. 1). A one-way ANOVA on the number of neuronal cells also revealed a significant effect of group [F(3,8)=9.52, $P<0.01$]. The effect of idebenone was significant at 100 nM and 1000 nM ($P<0.01$ and $P<0.05$, respectively). Aβ was highly toxic, and those neurons that did survive had an apparently reduced number of neurites which were shorter than those of control neurons. When idebenone was added to the culture medium with Aβ, the neuronal survival rate was significantly increased, and the neurons had a number of neurites as long as those seen in the control culture.

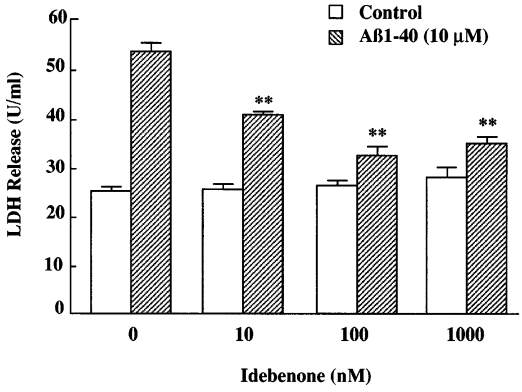

Fig. 1 Effects of idebenone on the Aβ1–40-induced neurotoxicity in rat hippocampal neurons. Hippocampal cells were grown for 6 days and exposed to Aβ1–40 (10 μM) with or without idebenone. Aβ1–40 was dissolved in PBS and incubated for 5 days at 37°C before application. Each value shows the mean LDH activity (± SEM) of three different cell wells determined 4 days after application of Aβ1–40 and/or idebenone. $^{**}P<0.01$, compared with the Aβ control culture (Dunnett's test)

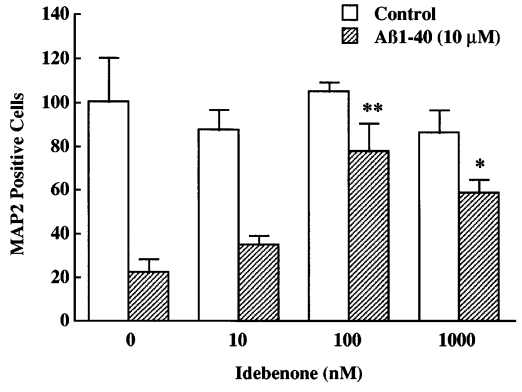

Fig. 2 Effects of idebenone on Aβ1–40-induced neurotoxicity in rat hippocampal neurons. The number of MAP2 positive cells were counted 5 days after the addition of Aβ1–40 and/or idebenone. Each value shows the mean number of MAP2 positive cells/3.91 mm^2 of three different cell wells (± SEM). $^{*}P<0.05$, $^{**}P<0.01$, compared with the Aβ control culture (Dunnett's test)

Discussion

Aβ deposition is crucial in the pathogenesis in the brain of AD patients. The Aβ protein is considered to be derived from the degradation of the amyloid precursor protein leading to amyloidogenic fragments and subsequent proteolysis. The peptide is then released and aggregates in the brain parenchyma into amyloid fibrillary deposits, and this causes the formation of a dense core of compacted senile plaque surrounded by dystrophic neurites and glial cells (Selkoe 1991). ROS have been shown to promote crosslinking and aggregation of Aβ and to enhance its neurotoxicity (Dyrks et al. 1992; Smith et al. 1995), and inversely, Aβ has been reported to generate ROS and to damage or kill neurons (Behl et al. 1994; Goodman and Mattson 1994). Recently, it has been shown that Aβ reduces the intracellular level of GSH (Müller et al. 1997) which may be closely related to generation of ROS.

Aβ1–40 showed delayed neuronal toxicity at the relatively high concentration of 10 μM. Several groups have reported a similar neurotoxicity at 10–50 μM in the hippocampal primary culture and PC12 cells (Behl et al. 1994; Goodman and Mattson 1994; Pike et al. 1993), which is consistent with our present study. Treatment with idebenone markedly protected the hippocampal neurons against the Aβ-induced neurotoxicity. It is likely that the neuroprotective effect of idebenone is due to its antioxidant action, because it has been reported that antioxidants including α-tocopherol suppressed the neurotoxicity (Behl et al. 1994; Goodman and Mattson 1994; Mattson and Goodman 1995; Miyamoto et al. 1989). The present study is consistent with these reports; however, others reported no protective effect of the same antioxidants (Pike et al. 1997). However, it has been suggested that these discrepancies may be related to differences in culture conditions including duration of cultures and the medium, and to the use of Aβ with different physical peptide properties including aggregation and conformation (Pike et al. 1997). In the same paper, these authors showed Aβ-induced lipid peroxidation and its inhibition by antioxidants; however, oxidative conditions which did not cause any damage on their own potentiated Aβ-induced neurotoxicity. Our culture system might be more oxidative than theirs because of the longer periods of culture in vitro. Moreover, we used some antioxidant (B27) in the preparation steps of cells. Thus, our dissociated culture might include some populations of neurons which are vulnerable to oxidative stress. Another difference is the concentration of Aβ used in the experiments. Pike et al. (1997) used 25 μM of Aβ, while we used 10 μM. Thus, in our study exposure to the presumably oxidative toxicity of Aβ was milder but longer; this difference in culture conditions might explain the divergent effects of antioxidants in the two studies.

Idebenone has been shown to potently inhibit lipid peroxidation in rat brain homogenates and the mitochondrial fraction; the effect becomes much stronger in the presence of the mitochondrial respiratory substrates (Suno and Nagaoka 1984). The finding that idebenone protects against the glutamate-induced cytotoxicity in N18-RE-105 cells at the low concentration of 100 nM (Miyamoto et al. 1989) is additional evidence that it exerts its neuroprotective effect at a low concentration in living cells with active mitochondrial respiratory activity. In this study, idebenone protected the hippocampal neurons against the Aβ1–40-induced neurotoxicity at concentrations of 10–1000 nM. The present results suggest that ROS may be extensively involved in β-amyloid-induced neurotoxicity, and idebenone may protect neurons against it.

Recent studies suggest that oxidative stress may play an important role in the pathogenesis of AD. Increased oxidative stress in the brain of AD patients is apparent from studies showing increased lipid peroxidation, increased carbonyl modification of proteins (Smith et al. 1995) and increased oxidation of mitochondrial DNA (mtDNA; Beal 1995). Recently, it has been demonstrated that Aβ causes oxidative damage of mtDNA (Bozner et al. 1997) and mitochondrial membrane damage (Bruce-Keller et al. 1998), suggesting a causative role of Aβ in mitochondrial dysfunction in AD. It was reported that the anti-oxidant action of idebenone is potentiated by mitochondria (Suno and Nagaoka 1984). This profile of idebenone may be advantageous to protect from Aβ-induced neurotoxicity. These findings suggest that idebenone may be useful for the treatment of AD. Indeed, the effects of idebenone on chronological changes in the symptoms of AD have been studied. Multicenter, placebo-controlled clinical studies with long-term administration of idebenone to AD patients showed that the treatment significantly improved cognitive and noncognitive function assessed by the Alzheimer's disease assessment scale (ADAS) scoring system, and indicated that idebenone clearly prevented the progression of clinical symptoms in patients with AD (Weyer et al. 1996). Although the contribution of Aβ in pathogenesis of AD is not yet understood, the neuroprotective effect of idebenone against Aβ-induced cytotoxicity observed in the present study might be, at least in part, related to its clinical therapeutic action in AD.

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