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Anxiolytic agent, dihydrohonokiol-B, recovers amyloid β protein-induced neurotoxicity in cultured rat hippocampal neurons

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Abstract

The effects of anxiolytic honokiol derivative, dihydrohonokiol-B (DHH-B), on amyloid β protein ($A\beta_{25–35}$, 10 nM)-induced changes in Cl^--ATPase activity, intracellular Cl^- concentration ($[Cl^-]_i$) and glutamate neurotoxicity were examined in cultured rat hippocampal neurons. DHH-B (10 ng/ml) recovered Aβ-induced decrease in neuronal Cl^--ATPase activity without any changes in the activities of Na^+/K^+-ATPase and anion-insensitive Mg^{2+}-ATPase. A $GABA_C$ receptor antagonist (1,2,5,6,-tetrahydropyridin-4-yl) methyl-phosphinic acid (TPMPA, 15 μM), inhibited the protective effects of DHH-B on Cl^--ATPase activity. DHH-B reduced Aβ-induced elevation of $[Cl^-]_i$ as assayed using a Cl^--sensitive fluorescent dye, and prevented Aβ-induced aggravation of glutamate neurotoxicity. These data suggest that DHH-B exerts the neuroprotective action against Aβ through $GABA_C$ receptor stimulation.

Keywords: Amyloid β protein; Dihydrohonokiol-B; Cl^--ATPase; $GABA_C$ receptor; Intracellular Cl^- concentration

Dihydrohonokiol (DHH-B; 3′-(2 propenyl)-5-(1,1′-biphenyl)-2,-4′-diol), a partially reduced derivative of honokiol isolated from magnolia bark [9], exhibits a potent anxiolytic activity in mice [6] and has been used in traditional Chinese medicine [10]. We previously showed that an ammonia-induced increase in intracellular chloride concentration (a model for ammonia neurotoxicity in hepatic coma) was recovered by DHH-B through $GABA_C$ receptor stimulation in primary cultured rat hippocampal neurons [5]. We also demonstrated that a $GABA_C$ receptor agonist suppressed ammonia-induced apoptosis in a $GABA_C$ receptor antagonist (TPMPA)-sensitive manner [12] and that $GABA_C$ receptor ρ subunits were expressed in neurons in cultures and slices of rat brain hippocampi [7]. Thus, DHH-B may exert $GABA_C$ stimulation activity to protect neuronal death.

We previously showed another model of pathophysiological neuronal death using amyloid β (Aβ) proteins, i.e. pathogenic peptides of Alzheimer's disease (AD) [11]. In this system, pathophysiological concentration (≤ 10 nM) of Aβ proteins, reduced neuronal Cl^--ATPase activity in cultured rat hippocampal neurons leading to the increases in the intraneuronal Cl^- concentration ($[Cl^-]_i$) as well as glutamate-induced neurotoxicity, the relative potency being $A\beta_{25–35} > A\beta_{1–42} > A\beta_{1–40}$. Since the activity of Cl^--ATPase, which is a candidate for outwardly directed active Cl^--transporter [3], was found to be reduced in the brains of AD [2], this in vitro model seems to be a relevant one for neuronal death in AD.

In the present study, we examined whether DHH-B had a protective effect against Aβ-induced neurotoxicity via $GABA_C$ receptor stimulation.

Hippocampal tissues of 19-day-old Wistar rat embryos were triturated in Ca^{2+}- and Mg^{2+}-free Hank's solution as described previously [4]. The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin G sulfate, 10% fetal calf serum and 10% horse serum. The cells were seeded in poly-L-lysine-coated plastic dishes at a density of 2.55×10^5 cells/cm^2. After incubation for 2 days, the

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cells were exposed to 5 μM adenine-9β-arabinofuranoside (Ara-A) in modified Eagle's medium (MEM) supplemented with 2 mM L-glutamine and 5% horse serum for 4 days [1]. $A\beta_{25–35}$ and other reagents were applied for 2 days from the 8th day of culture. For monitoring glutamate excitotoxicity, the cells were exposed to glutamate (10 μM, 10 min) on the 10th day of culture and assayed for cell viability after another 2-day culture in the media with or without Aβ and other reagents.

Plasma membrane-rich fractions were prepared on the 10th day of culture, as described previously [11]. Briefly, the cultured cells were homogenized in ice-cold buffer solution containing 0.25 M sucrose, 1 mM EDTA–Tris (pH 7.4), 12.5 mM Tris–2-(*N*-morpholino)-ethanesulfonic acid (Tris–Mes, pH 7.4), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 50 units/ml aprotinin, and centrifuged (10,000 × *g*, 15 min; 100,000 × *g*, 20 min). The pellets were suspended in 2 mM EDTA–Tris (pH 7.4), stirred for 30 min, and centrifuged (100,000 × *g*, 20 min). The resulting pellets were resuspended in 2 mM EDTA–Tris (pH 7.4) and used as plasma membrane-rich fractions. The protein concentration was determined by the method of Lowry et al. [8].

ATPase activities were determined by spectrophotometric measurement of the inorganic phosphate liberated. The incubation was carried out for 15 min at 37 °C in 200 μl reaction buffer containing 100 mM Tris–Mes (pH 7.4), 1 mM EDTA–Tris, 100 mM NaCl, 10 mM KCl, 6 mM magnesium acetate, 6 mM ATP–Tris (pH 7.4), 2 mM NaN_3 and 3–6 μg membrane protein with or without 1 mM ouabain and/or 0.3 mM ethacrynic acid (EA). The reaction was terminated by the addition of 10% trichloroacetic acid. Na^+/K^+-ATPase was calculated by subtracting the ATPase activity in the presence of 1 mM ouabain from the total ATPase activity. The activity in the presence of 1 mM ouabain and 0.3 mM EA was designated as anion-insensitive Mg^{2+}-ATPase. The difference between ATPase activities in the presence and absence of 0.3 mM EA was designated as Cl^--ATPase activity.

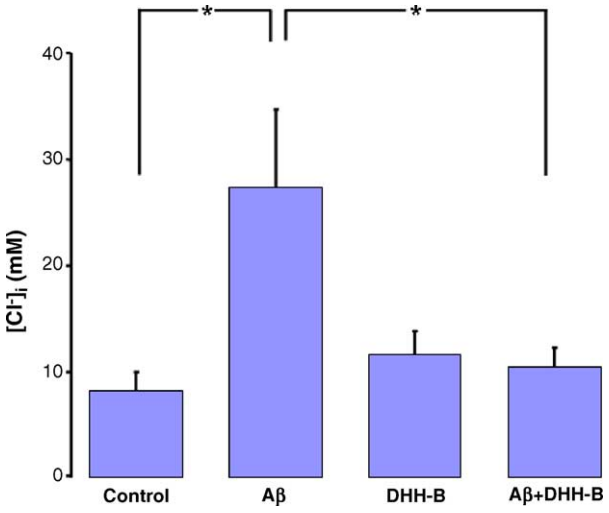

Fig. 2. Effects of DHH-B on the $A\beta_{25–35}$-induced changes in $[Cl^-]_i$ in cultured rat hippocampal neurons. Neurons on the 8th day of culture were exposed to 10 nM $A\beta_{25–35}$ with or without 10 ng/ml DHH-B for 2 days. $[Cl^-]_i$ of pyramidal cell-like neurons was assayed fluorometrically using a Cl^--sensitive fluorescent dye (MQAE) on the 10th day of culture ($n = 8–14$, * $P < 0.05$).

For the measurement of $[Cl^-]_i$, dissociated hippocampal cells were cultured on poly-L-lysine-coated coverslips in plastic dishes, and treated as described above. The cells were washed with modified Krebs–HEPES buffer solution (pH 7.3) containing 128 mM NaCl, 2.5 mM KCl, 2.7 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM HEPES and 16 mM glucose, exposed to 5 mM *N*-(6-methoxyquinolyl)-acetoethyl ester (MQAE), a Cl^--sensitive fluorescent dye, in the same buffer solution for 1 h at 37 °C, and then washed with a dye-free buffer solution. Fluorescence intensity of a single pyramidal cell-like

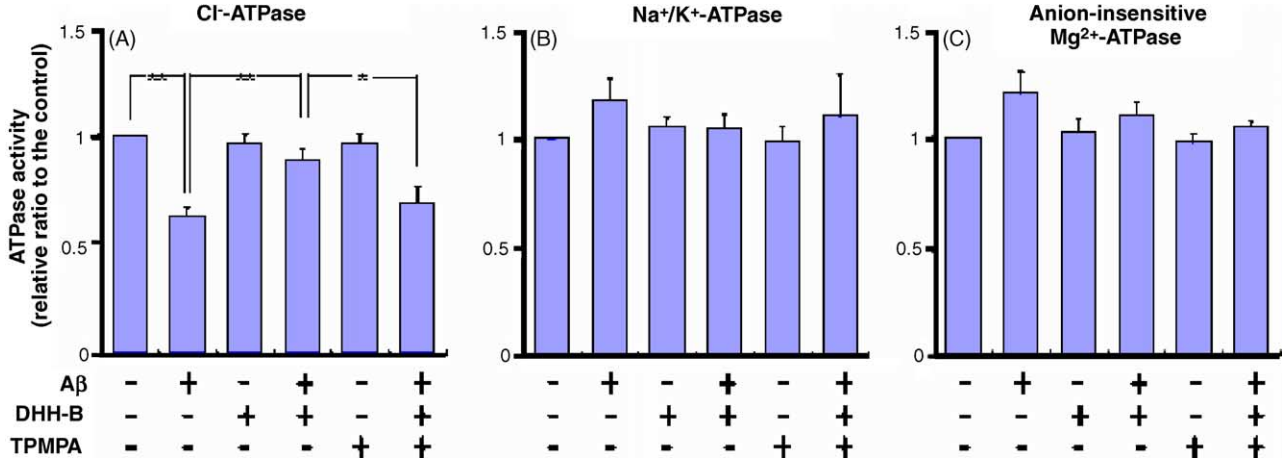

Fig. 1. Effects of dihydrohonokiol-B (DHH-B) on the $A\beta_{25–35}$-induced changes in Cl^--ATPase in cultured rat hippocampal neurons. Neurons on the 8th day of culture were exposed to 10 nM $A\beta_{25–35}$ with or without 10 ng/ml DHH-B and/or 15 μM TPMPA for 2 days. Cell membranes were prepared and assayed for ATPase activities of Cl^--ATPase, Na^+/K^+-ATPase and anion-insensitive Mg^{2+}-ATPase on the 10th day of culture ($n = 5–14$, * $P < 0.05$, ** $P < 0.01$). Mean ± S.E. values of each control ATPase activity (μmol Pi/mg protein/h) were: Cl^--ATPase, 4.8 ± 0.48; Na^+/K^+-ATPase, 5.3 ± 0.3; anion-insensitive Mg^{2+}-ATPase, 18.2 ± 1.0, respectively.