Chemico-Biological Interactions 203 (2013) 365–370

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Bis(12)-hupyridone, a novel acetylcholinesterase inhibitor, protects against glutamate-induced neuronal excitotoxicity via activating α7 nicotinic acetylcholine receptor/phosphoinositide 3-kinase/Akt cascade

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ARTICLE INFO

Article history:
Available online 16 October 2012

Keywords:
Bis(12)-hupyridone
Glutamate
α7nAChR
NMDA receptor
Akt

ABSTRACT

Bis(12)-hupyridone (B12H), derived from the Chinese medicinal component huperzine A, was originally designed as a novel acetylcholinesterase (AChE) inhibitor. In this paper, we report that B12H (24-h pretreatment) effectively blocked glutamate-induced neuronal excitotoxicity in cerebellar granule neurons (CGNs). However, the huge discrepancy between the EC_{50} value and IC_{50} value of B12H, to protect against neuronal toxicity (0.09 μM) and to block the NMDA receptor (21.8 μM) respectively, suggests that the neuroprotection of B12H might be not primarily due to the blockade of the NMDA receptor. Pretreatment by specific antagonists of alpha7-nicotinic acetylcholine receptor (α7nAChR), but not muscarinic acetylcholine receptor (mAChR) or α4β2nAChR, decreased the neuroprotection of B12H. The neuroprotection of B12H could also be abolished by the pretreatment of specific PI3-K inhibitors. Furthermore, B12H restored the suppressed activation of the Akt pathway caused by glutamate as evidenced by the decreased expressions of pSer473-Akt and pSer9-GSK3β. All these results suggest that B12H substantially protected CGNs against glutamate-induced neuronal excitotoxicity via activating α7nAChR/PI3-K/Akt cascade.

1. Introduction

Glutamate-induced neuronal excitotoxicity plays an important role in chronic neurodegenerative disorders such as Alzheimer's disease (AD) [3]. An abnormal glutamate efflux causes substantial neurological damage in these diseases [4]. Elevation of glutamate level causes hyperactivity of the N-methyl-D-aspartate (NMDA) receptor, leading to neuronal excitotoxicity [4]. Therefore, moderate antagonists of NMDA receptor could effectively block glutamate-induced neuronal excitotoxicity and be used in the treatment of AD. Recently, many studies have shown that stimulating certain types of nicotinic acetylcholine receptors (nAChR) also protects against glutamate-caused neuronal excitotoxicity [1,22]. Nicotine protected cortical neurons against glutamate neurotoxicity via activating the α4β2 and α7nAChRs [23]. Donepezil and galantamine, acetylcholinesterase (AChE) inhibitors used in the clinical treatment of AD, were also found to prevent glutamate-induced neuronal loss via stimulation of the α7nAChR [22]. The activation of phosphoinositide 3-kinase (PI3-K)/Akt signal transduction was indicated to contribute to the neuroprotective effects of stimulated nAChRs, especially α7nAChR [1]. Activation of nAChR increases the level of phosphorylated Akt, an effector of PI3-K, which further inhibits the activity of glycogen synthase kinase 3β (GSK3β), increases the internalization of NMDA receptor, and leads to neuroprotection [2,7,11].

Bis(12)-hupyridone (B12H) is a novel synthetic dimeric AChE inhibitor derived from the natural compound huperzine A (Fig. 1) [6]. Huperzine A was originally isolated from the Chinese medical herb *Huperzia serrata*. Owing to its beneficial effects to neurodegenerative disorders, huperzine A has been approved for the treatment of AD in China [26]. We have reported that B12H could readily cross the blood brain barrier of mice after peritoneal injection and inhibit rat brain AChE at a higher potency than huperzine A *in vitro* [14,27]. We have also demonstrated that B12H prevented H_2O_2-induced

Abbreviations: AChE, acetylcholinesterase; AD, Alzheimer's disease; B12H, bis(12)-hupyridone; CGNs, cerebellar granule neurons; DHβE, dihydro-β-erythroidine; GSK3β, glycogen synthase kinase 3β; mAChR, muscarinic acetylcholine receptor; MLA, methyllycaconitine; NMDA, *N*-methyl-D-aspartate; PI3-K, phosphoinositide 3-kinase; α7nAChR, alpha7-nicotinic acetylcholine receptor.

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http://dx.doi.org/10.1016/j.cbi.2012.10.003

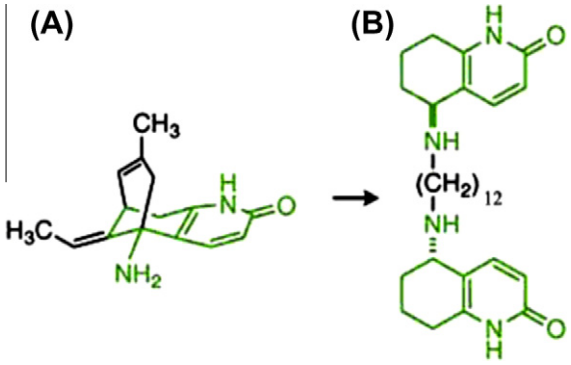

Fig. 1. The chemical structures of huperzine A (A) and B12H (B).

apoptosis in primary cerebellar granule neurons (CGNs) and promoted neuronal differentiation in neural stem cells [9].

It has been reported that huperzine A protected against glutamate-induced neuronal death in enriched neuronal culture [25]. The current study was undertaken to study the effects and underlying mechanisms of B12H in preventing glutamate-induced neuronal excitotoxicity using primary CGNs. We demonstrated that B12H protected against glutamate-induced neuronal excitotoxicity via activating the α7nAChR/PI3-K/Akt cascade.

2. Materials and methods

2.1. Chemicals and reagents

B12H was synthesized as we previously described [6]. B12H was dissolved in Milli-Q water at a concentration of 3 mM and stored frozen at −20 °C. It was further diluted with Milli-Q water before use. Unless otherwise stated, all media and supplements used for cell cultures were purchased from Invitrogen (Carlsbad, CA, USA). LY294002, wortmannin, atropine, tubocurarine, mecamylamine, methyllycaconitine (MLA), and dihydro-β-erythroidine (DHβE) were obtained from Sigma Chemicals (St. Louis, MO, USA). Antibodies against Akt, GSK3β, phospho-Akt and phospho-GSK3β were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibody against β-actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Primary cell cultures

All animal experiments were conducted according to the ethical guidelines of the Animal Care Facility, The Hong Kong Polytechnic University. CGNs were prepared from 8-day-old Sprague–Dawley rats as described in our previous publication [13]. Briefly, neurons were plated at a density of 2.0×10^6 cells/ml in basal modified Eagle's medium containing 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cytosine arabinoside (10 μM) was added to the culture medium 24 h after plating to limit the growth of non-neuronal cells.

Primary hippocampal neurons were obtained from 18-day-old Sprague–Dawley rat embryos as previously described [10,13]. Briefly, the hippocampal neurons were plated at a density of 4×10^5/ml cells. Cells were maintained in Neuro-basal/B27 medium containing 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Half-changes of the medium were done twice weekly.

2.3. MTT assay

The percentage of surviving neurons in the presence of B12H and/or glutamate was estimated by determining the activity of mitochondrial dehydrogenases with 3(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay [13]. The absorbance of the samples was measured at a wavelength of 570 with 655 nm as the reference. Unless otherwise indicated, the extent of MTT conversion in the cells exposed to glutamate is expressed as a percentage of the control.

2.4. Whole-cell electrophysiological analysis

Cultured hippocampal neurons were used for whole-cell patch clamp recording 7–15 days after plating [18]. Before each experiment, the culture medium was removed, the cells were thoroughly rinsed and continuously superfused with a solution containing (in mM):150 NaCl, 5 KCl, 0.25 $CaCl_2$, 10 glucose, 0.001 glycine, 0.001 tetrodotoxin, 0.01 (−)-bicuculline methiodide, and 10 HEPES (the pH was adjusted to 7.4 with NaOH, and the osmolarity was adjusted to 340 mOsm with sucrose). A low concentration of Ca^{2+} was used to minimize the calcium-dependent desensitization of NMDA-activated current. Pipettes pulled from borosilicate glass had resistances of 2–4 MΩ when filled with a pipette solution containing (in mM): 140 CsCl, 10 EGTA, 10 HEPES, and 5 MgATP with pH 7.3 and 315 mOsm in osmolarity. The holding potential was set at −60 mV except when otherwise indicated. Data were acquired using pClamp 9.0 software (Axon Instruments). Currents were filtered at 2 kHz and digitized at 5 kHz.

2.5. Western blot assay

Western blot analysis was performed as previously described [12]. In brief, neurons were harvested in a cell lysis buffer. The protein (30 μg) was separated on a 10% SDS–polyacrylamide gel. Blocking was performed onto polyvinyldifluoride membranes. Proteins were detected using primary antibodies. After incubation at 4 °C overnight, signals were obtained by using a secondary antibody. Blots were developed using an ECL plus kit (Amersham Bioscience, Aylesbury, UK), exposed to Kodak autoradiographic films and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

2.6. Statistical analysis

Results are expressed as mean ± SEM. Analysis of variance (ANOVA) followed by a Dunnett's test or Tukey's test was used for statistical comparisons. Levels of $p < 0.05$ were considered to be of statistical significance.

3. Results

3.1. B12H prevents glutamate-induced neuronal excitotoxicity in CGNs

At 8 DIV, CGNs were pretreated with B12H, donepezil or huperzine A for 24 h, and then exposed to 100 μM glutamate for another 24 h. Cell viability was measured by MTT assay, and the EC_{50} values were calculated relative to the cell viability of untreated control (100%). We found that B12H prevented glutamate-induced neuronal death in CGNs at an EC_{50} value of 0.09 μM, which is about 10 times more potent than that of huperzine A (Fig. 2). In this model, donepezil also protected against glutamate-induced neuronal death, that is in agreement with previous publication [24]. Treatments of B12H (0.003–2 μM), donepezil (0.03–10 μM), or huperzine A (0.03–30 μM) alone for 48 h showed no cell proliferative or cytotoxic effects (data not shown).