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2 HARPAGOSIDE AMELIORATES THE AMYLOID-β-INDUCED COGNITIVE 3 IMPAIRMENT IN RATS VIA UP-REGULATING BDNF EXPRESSION AND 4 MAPK/PI3K PATHWAYS

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12 **Abstract—So far, no effective disease-modifying therapies for Alzheimer's disease (AD) aiming at protecting or reversing neurodegeneration of the disease have been established yet. The present work aims to elucidate the effect of Harpagoside (abbreviated HAR), an iridoid glycosides purified from the Chinese medicinal herb *Scrophularia ningpoensis*, on neurodegeneration induced by β-amyloid peptide (Aβ) and the underlying molecular mechanism. Here we show that HAR exerts neuroprotective effects against Aβ neurotoxicity. Rats injected aggregated $A\beta_{1-40}$ into the bilateral hippocampus displayed impaired spatial learning and memory ability in a Y-maze test and novel object recognition test, while HAR treatment ameliorated $A\beta_{1-40}$-induced behavioral deficits. Moreover, administration of HAR increased the expression levels of brain-derived neurotrophic factor (BDNF) and activated the extracellular-regulated protein kinase (ERK) and the phosphatidylinositol 3-kinase (PI3-kinase) pathways both in the cerebral cortex and hippocampus of the $A\beta_{1-40}$-insulted rat model. Furthermore, in cultured primary cortical neurons, $A\beta_{1-42}$ induced significant decrease of choline acetyltransferase (ChAT)-positive neuron number and neurite outgrowth length, both of which were dose dependently increased by HAR. In addition, HAR pretreatment also significantly attenuated the decrease of cell viability in $A\beta_{1-42}$-injured primary cortical neurons. Finally, when K252a, an inhibitor of Trk tyrosine kinases, and a BDNF neutralizing antibody were added to the culture medium 2 h prior to HAR addition, the protective effect of HAR on $A\beta_{1-42}$-induced neurodegeneration in the primary cortical neuron was almost inhibited. Taken together, HAR exerting neuroprotection effect and ameliorating learning and memory deficit appear to be associated, at least in part, with up-regulation of BDNF content as well as activating its downstream signaling pathways e.g., MAPK/PI3K pathways. It raises the possibility that HAR has potential to be a therapeutic agent against AD.**
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Key words: brain-derived neurotrophic factor (BDNF), Alzheimer's disease, learning and memory deficits, p-ERK1/2, p-AKT, Aβ.

13

14 INTRODUCTION

15 Alzheimer's disease (AD) is a common age-related 16 neurodegenerative disorder, characterized clinically by 17 progressive cognitive deficit starting from memory 18 impairment to cognitive deterioration (Albert et al., 19 2011). Intracellular neurofibrillary tangles (NFT) aggre-20 gated by hyperphosphorylated tau and extracellular senile 21 plaques composed of β-amyloid (Aβ) protein are the 22 pathological hallmarks of AD (Giacobini and Becker, 23 2007). Currently, acetylcholinesterase (AChE) inhibitors 24 and N-methyl-D-aspartate (NMDA) receptor antagonists 25 are the mainstay of clinically therapeutic regimens for 26 AD; however they only alleviate symptoms but fail to halt 27 its progression (Herrmann et al., 2011; Yiannopoulou and 28 Papageorgiou, 2013). Thus, it is imperative to develop 29 novel and effective medications aiming at delaying the 30 onset and progression of AD that go beyond AChEIs 31 and NMDA antagonists.

32 Injection of Aβ into hippocampus of rat which can 33 mimic the pathogenesis of AD and trigger cognitive 34 impairments has been frequently served as a useful 35 experimental animal model for AD (Shin et al., 1997; 36 Chacon et al., 2004; Tang et al., 2008; Wu et al., 2013). 37 Brain-derived neurotrophic factor (BDNF), a member of 38 the neurotrophin family, activates its downstream signal-39 ing pathways including extracellular signal-related kinase 40 (ERK1/2) and phosphoinositide 3-kinase (PI3K)/Akt 41 through binding to its receptor tyrosine receptor kinase 42 B (TrkB). Thus, BDNF plays pivotal roles in neuronal 43 growth, survival, synaptic plasticity as well as learning 44 and memory (Yamada et al., 2002; Almeida et al., 45 2005). It has been also well documented that BDNF pro-46 tein or mRNA are significantly reduced in the brain of 47 patients with AD (Connor et al., 1997; Hock et al., 2000; 48 Michalski and Fahnestock, 2003; Peng et al., 2005) and

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Abbreviations: AChE, acetylcholinesterase; AD, Alzheimer's disease; Aβ, β-amyloid; BDNF, brain-derived neurotrophic factor; ChAT, choline acetyltransferase; DAB, 3,3′-diaminobenzidine; DMEM, Dulbecco's Modified Eagle Medium; ERK, extracellular-regulated protein kinase; HAR, Harpagoside; HPMC-Na, hydroxypropyl methyl cellulose; NMDA, N-methyl-D-aspartate; PI3-kinase, phosphatidylinositol 3-kinase; RI, recognition index; TrkB, tyrosine receptor kinase B.

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AD animal models (Christensen et al., 2008; Peng et al., 2009; Caccamo et al., 2010; Shin et al., 2014). Furthermore, accumulating evidence supports that therapeutic strategies targeting ameliorating AD pathology and ameliorating cognitive impairment in AD models are associated with BDNF up-regulation (Blurton-Jones et al., 2009; Caccamo et al., 2010; Iwasaki et al., 2012). BDNF infusion or gene delivery to *in vivo* AD models ameliorates cognitive impairment (Nagahara et al., 2009, 2013; Iwasaki et al., 2012). Nevertheless, BDNF possesses the intrinsic drawbacks, such as its poor blood–brain barrier penetration or BDNF gene/protein delivery inducing mutagenesis or toxicity, thus natural products and small molecules that can induce endogenous BDNF expressions are under investigation (Zuccato and Cattaneo, 2009; Shin et al., 2014).

Harpagoside (HAR) (abbreviated as HAR, Fig. 1A), a derivative of catalpol (Fig. 1B), is an iridoid glycosides from *Scrophularia ningpoensis*. Our previous study has confirmed that catalpol, an iridoid glucoside in *Rehmannia glutinosa*, could improve the capability of learning and memory of neurodegenerative animals as well as increase BDNF expression in Aβ-induced AD model (Wang et al., 2009). Notably, HAR has been reported to exert neuroprotective effect against glutamate-induced neurotoxicity in primary cortical neurons (Kim et al., 2003) and alleviate memory deficit induced by scopolamine in mice through antioxidant mechanisms (Jeong et al., 2008). Our previous study has demonstrated that HAR could enhance GDNF contents as well as motor function of MPTP-lesioned mice *in vivo* (Sun et al., 2012). However, the precise action mechanism of HAR is still poorly understood. To this end, we would like to explore whether HAR could exert neuroprotective effect against Aβ-induced AD model and the underlying molecular mechanisms of these effects.

In the study, we aimed to investigate the neuroprotective effect of HAR on Aβ model. We firstly attempted to determine the impact of HAR on the neurotoxic effects of $A\beta_{1-42}$-induced primary cortical neurons. We then evaluated protective effects of HAR against Aβ-induced neuronal cell death, and ChAT-positive neuron number and outgrowth length *in vitro*. In parallel, we also showed whether HAR could improve the learning and memory ability of rat using AD models and induce BDNF expression. Lastly, we examined whether the effect of HAR was related to BDNF' expression and function. Thus, we indeed discover HAR with a potential neuroprotective effect of HAR against Aβ intoxication.

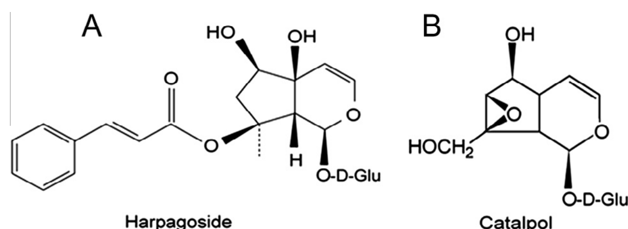

Fig. 1. Chemical structures of Harpagoside (A) and Catalpol (B).

EXPERIMENTAL PROCEDURES

Materials

HAR with a purity of over 98% determined by HPLC was supplied by Shanghai Tauto Biotechnology Co (Shanghai, China). $A\beta_{1-42}$ and $A\beta_{42-1}$ was purchased from invitrogen. $A\beta_{1\text{-}40}$ was from Sigma-Aldrich (USA). Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) and supplement B27 were obtained from Gibco (Grand Island, NY, USA). Methyl thiazol tetrazolium (MTT) and poly-L-lysine were acquired from Sigma (St. Louis, MO, USA). Hydroxypropyl methyl cellulose (HPMC-Na) was acquired from Sigma (St. Louis, MO, USA). SABC kit was bought from Boster Bioengineering Co. (Wuhan, China). Sheep anti-BDNF polyclonal neutralizing antibody, rabbit anti-rat choline acetyltransferase (ChAT) antibody, biotin-labeled sheep anti-rabbit IgG secondary antibody and 3,3′-diaminobenzidine (DAB) were from Chemicon (Temecula, CA, USA). BDNF ELISA (Emax. Immunoassay) kit was acquired from Promega (Madison, WI, USA). Mouse anti-phospho-ERK1/2 (Thr202/Tyr204), rabbit anti-ERK1/2, rabbit anti-phospho-AKT (Ser473) and rabbit anti-AKT were obtained from Cell Signaling Technology. K252a was from Biomol. RIPA lysis buffer, BCA protein assay kit and ECL were purchased from Beyotime.

Intrahippocampal $A\beta_{1-40}$ injection and drug treatment

Male Sprague–Dawley rats (8 weeks old, 253.64 ± 1.52 g, from Shanghai SIPPR-BK Laboratory Animal Company) were housed two animals per cage and kept on a 12-h light–dark cycle in standard conditions with temperature and relative humidity set at 22 ± 2 °C and 55% ± 15% respectively, with free access to pellet diet and water. All procedures were conducted in accordance to the NIH Guide for the Care and Use of Experimental Animals and were approved by the Shanghai Jiaotong University Animal Ethic Committee.

After a one-week period of acclimatization, all rats were randomly divided into four groups: sham control group, $A\beta_{1-40}$-induced model group, Aβ + HAR 5 mg/kg group, and Aβ + HAR 15 mg/kg group.

The procedure of the AD model was modified slightly from previous study (Wu et al., 2013). $A\beta_{1-40}$ was firstly dissolved in sterile 0.9% saline at a concentration of 5 μg/μl and incubated at 37 °C for 4 days to allow aggregation before intrahippocampal injection (Han et al., 2011). Rats were weighed and deeply anesthetized with 10% chloral hydrate (350 mg/kg, i.p.), fixed into a stereotaxic apparatus. A burr hole was drilled through the skull above the bilateral hippocampal coordinates (anterior–posterior (AP) = −3.0 mm, medial-lateral (ML) = ± 2.0 mm from the bregma and dorsal-ventral (DV) = 3.5 mm from the skull surface) according to stereotaxic atlas (Paxinos and Watson, 1986) and 2 μl containing 10 μg of $A\beta_{1-40}$ was subsequently injected over 5 min through a microsyringe into the hole and the needle was left for additional 5 min before withdrawal.

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