Potential therapeutic targets of huperzine A for Alzheimer’s disease and vascular dementia

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**A B S T R A C T**

Huperzine A (HupA), a novel **Lycopodium** alkaloid isolated from Chinese folk medicine **Huperzia serrata** (*Qian Ceng Ta*), is a potent, selective and well-tolerated inhibitor of acetylcholinesterase (AChE). It has been proven to significantly improve the learning and memory impairment in Alzheimer’s disease (AD) and vascular dementia (VaD) patients in China. Interestingly, our recent data indicate that HupA also possesses other protective functions. This paper will give an overview on the protective effects of HupA, which includes regulating β-amyloid precursor protein (APP) metabolism, protecting against Aβ-mediated oxidative stress, apoptosis and mitochondrial dysfunction, as well as anti-inflammation. The multiple neuroprotective effects of HupA might yield additional beneficial effects in AD and VaD therapy.

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Since the etiology of AD involves multiple mechanisms, drugs that possess multiple targets besides the cholinergic function will be expected to be more effective in AD therapy. Interestingly, recent data indicate that HupA has multiple protective effects aside from its AChE inhibition [8]. Consistent with our result, HupA was also shown to protect motor neuron cultures from toxin (H2O2, staurosporine, thapsigargin et al.)-induced cell death [9]. These effects will be briefly reviewed in this paper, mainly focused on β-amyloid (Aβ) related pathogenesis and inflammation.

1. Effects of huperzine A on β-amyloid-related pathogenesis

AD is characterized by two main pathological hallmarks: extracellular deposition of the Aβ in senile plaques, and the appearance of intracellular neurofibrillary tangles (NFT) [10]. A growing body of evidence indicates that Aβ is central to the pathology of AD and is likely to start this intractable neurodegenerative disorder [11–15]. It has been proven that Aβ treatment can generate oxidative stress, apoptosis and mitochondria dysfunction both in vivo and in vitro, which could eventually trigger a state of neurotoxicity and cell death [12–15]. Hence, modifying β-amyloid precursor protein (APP) processing or attenuating Aβ-mediated neurotoxicity would be expected to be another strategy for alleviating AD pathology.

1.1. Effects of huperzine A on APP processing

Aβ is produced mainly by two pathways: the amyloidogenic pathway, which is mediated by β-secretase and γ-secretase, creates Aβ peptide; the non-amyloidogenic pathway, which is mediated by α-secretase and γ-secretase, cleaves APP within Aβ sequence and releases a soluble secretory amyloid precursor protein (sAPPα) [16,17]. sAPPα has been reported to exhibit neuroprotective effects including promotion of cell proliferation and neurite outgrowth, as well as prevention of intracellular calcium accumulation [18], all of which may delay AD progression.

In agreement with previous data that AChE inhibitors can affect APP processing [19,20], our recent data indicated that HupA stimulated sAPPα release (Fig. 2) in intracerebroventricular Aβ-infused rats and human embryonic kidney 293 Swedish mutant cells [21], similar results were also shown by other group [22]. Further study [23] indicated that up-regulation of sAPPα level by HupA was attenuated by antagonists of muscarinic acetylcholine receptor (mAChR), especially M1, through activating protein kinase C (PKC)-mediated pathway. Our results are consistent with previous finding that M1 is involved in the processing of APP by activating α-secretase mediated cleavage [24,25]. Moreover, our studies found that HupA can activate the phosphorylation of mitogen activated protein kinase (MAPK) and accordingly, partly restored MAPK inhibitor – PD98059 – caused decrease in sAPPα release. Taken together, our results suggest that activated M1-mAChR/PKC pathway and MAPK signaling, which are the two main pathways involved in M1-mediated APP processing [24], are involved in the effects of HupA on enhancing the sAPPα secretion [23]. In addition, the effect of HupA on MAPK pathway was also shown in PC12 cells, SH-SYSY cells, and cultured rat cortical astrocytes, as well as in regulating nerve growth factor (NGF) signaling [26–28].
1.2. Antioxidative effect

Our previous studies found that HupA significantly alleviated Aβ-induced neurotoxicity in PC12 cells [29] (Fig. 3), NG-108 cells [30], primary cortical neuron cultures [14], and in rats [15]. Similar as HupA, other AChEIs also exert neuroprotective effects on Aβ-induced injuries. Donepezil significantly reduced the LDH efflux induced by Aβ1–40 at 100 nmol/L and above [31]; galantamine showed neuroprotective effects on Aβ1–40 and thapsigargin-induced cell death in the human neuroblastoma cell line SH-SY5Y, as well as in bovine chromaffin cells [32]. Moreover, in vivo data also found that donepezil, tacrine, rivastigmine and galantamine could improve Aβ25–35-induced memory deficiency in mice [33,34]. The neuroprotective effects of those AChEIs on Aβ neurotoxicity maybe helpful to slow down the progression of AD.

One of pathways that mediate Aβ toxicity is through oxidative stress. Under Aβ exposure, cells will generally produce reactive oxygen species (ROS), which can lead to damage or destruction of a variety of tissues, and consequently cause lipid peroxidation, oxidation of proteins, and damage of DNA. The major antioxidants in cells include glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT), along with other nonenzymatic antioxidants, such as cysteine, ascorbate and α-tocopherol. The combined action of these antioxidants provides a repair mechanism for oxidized membrane components. Aβ exposure significantly decreased activity of GSH-Px and increased level of the lipid peroxidation product—malondialdehyde (MDA). Pre-incubation of cells with HupA prior to Aβ exposure was found to enhance cell survival and activities of antioxidant enzymes including GSH-Px, SOD and CAT, and decrease MDA production [29] (Table 1). Similar antioxidative effects of HupA were also shown in hydrogen peroxide-induced cell injury model [35], chronic hypo-perfusion rat model and in aged rats [36]. Our study found that the some of antioxidant effects of HupA did not show dose dependency, it came up with a bell-shaped dose–response curve. It is not a rare phenomenon in evaluating the neuroprotective effects on different neurotoxins-induced injuries, similar result was also reported by other groups [22,31,32,37,38].

1.3. Anti-apoptosis effect

Other than oxidative stress, apoptosis is also believed to be an important contributor to the progression of AD and mediating Aβ neurotoxicity. There is considerable evidence showing that Aβ can activate intracellular apoptosis pathways [39,40], since application of Aβ to cells could up-regulate apoptotic relative protein Caspase-3 [41] and annexin [42], and eventually causes apoptosis [43]. The cellular commitment to apoptosis is regulated by the Bcl-2 family of proteins. Current understanding of the bcl-2 gene family indicates that the interactions and relative abundance of Bcl-2 and Bax can modulate the propensity of a cell to undergo apoptotic death [44], and that increased expression of P53 and Bax is associated with the initiation of apoptosis [45]. Through both in vivo and in vitro studies, HupA was shown to significantly alleviate Aβ-induced apoptotic changes including DNA laddering, cell shrinkage, generation of nuclear apoptotic bodies, and TUNEL positive staining (Fig. 4) [14,15]. These anti-apoptotic effects may through reversing the down-regulation of Bcl-2 level and the up-regulation of Bax and P53 levels (Fig. 5) [15]. Moreover, similar anti-apoptotic effects of HupA were shown in hydrogen peroxide and staurosporine-induced apoptosis cell models [46,47].

The abnormal changes in Bcl-2, Bax and P53 expression will induce the release of cytochrome c, which binds to Apaf-1 and polymerizes into an oligomer known as apoptosome. The apoptosome activates Caspase-9, which in turn activates the apoptotic executive protein, Caspase-3, and finally leads to apoptosis [48,49]. HupA was found to attenuate the increase of Caspase-3 activity induced by Aβ in cultured primary cortical neurons [14], these effects were further confirmed in apoptosis models of serum deprivation and staurosporine stimulation [47,50].

1.4. Improving mitochondrial function

Mitochondria are the powerhouse of the cell, and functioning mitochondria are required for maintaining normal physiological status. In contrast, the mitochondrial dysfunction is considered as one of the key intracellular lesions associated with the pathogenesis of AD. In the mitochondrial compartment, accumulation of Aβ [51] and direct interaction of Aβ with Aβ-binding alcohol dehydrogenase (ABAD) [52] have been proven to play a causative role in impairing mitochondrial physiological functions. Consistent with previous studies [53,54], we found that Aβ exposure caused overproduction of ROS and serious damage to activities of key components of mitochondrial respiratory chain and the tricarboxylic acid (TCA) cycle. Aβ exposure then caused a loss of cellular ATP and the collapse
Table 1  
Effects of HupA on 1.0 μM Aβ25–35-induced antioxidant enzyme activities and level of lipid peroxidation in PC12 cells

<table>
<thead>
<tr>
<th></th>
<th>% of control</th>
<th>Catalase</th>
<th>GSH-Px</th>
<th>SOD</th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (units/mg protein)</td>
<td>100 ± 8.2 (17.1 ± 1.4)</td>
<td>100 ± 10.5 (9.5 ± 1.0)</td>
<td>100 ± 4.4 (3.2 ± 0.14)</td>
<td>100 ± 6.9 (0.145 ± 0.01)</td>
<td></td>
</tr>
<tr>
<td>HupA (1.0 μM)</td>
<td>117.5 ± 19.4</td>
<td>113.7 ± 15.7</td>
<td>109.4 ± 17.4</td>
<td>97.4 ± 13.8</td>
<td></td>
</tr>
<tr>
<td>Aβ25–35 (1.0 μM)</td>
<td>49.9 ± 5.5a</td>
<td>56.0 ± 3.7a</td>
<td>141 ± 22.4a</td>
<td>164 ± 11.0a</td>
<td></td>
</tr>
<tr>
<td>Aβ25–35 + HupA (0.01 μM)</td>
<td>58.6 ± 3.7</td>
<td>74.4 ± 9.4</td>
<td>122 ± 9.7</td>
<td>141 ± 15.0</td>
<td></td>
</tr>
<tr>
<td>Aβ25–35 + HupA (0.1 μM)</td>
<td>68.9 ± 7.7</td>
<td>80.1 ± 4.4</td>
<td>114 ± 8.8</td>
<td>124 ± 7.1</td>
<td></td>
</tr>
<tr>
<td>Aβ25–35 + HupA (1 μM)</td>
<td>82.0 ± 14.8a</td>
<td>85.2 ± 7.0a</td>
<td>104 ± 72</td>
<td>117 ± 12.0a</td>
<td></td>
</tr>
<tr>
<td>Aβ25–35 + HupA (10 μM)</td>
<td>66.6 ± 7.2</td>
<td>79.6 ± 6.9</td>
<td>122 ± 8.9</td>
<td>130 ± 13.0</td>
<td></td>
</tr>
</tbody>
</table>

*PC12 cells were exposed to 1.0 μM Aβ25–35. HupA was added 2 h prior to Aβ25–35 addition and the levels of antioxidants and MDA were measured 48 h later. Data were means ± S.E.M. expressed as percent of the control value. #P < 0.01 vs. control; *P < 0.01 vs. Aβ25–35 group. Data from [29].

Fig. 4. Apoptotic cell death induced by i.c.v. infusion of Aβ1–40 in rats. DNA fragmentation was observed using the TUNEL methods. (A) Vehicle–treated rats. (B) Aβ1–40–treated rats. (C) Rats treated with Aβ1–40 and daily administration of HupA (0.2 mg/kg, i.p.) for 12 consecutive days. Scale bar = 5 μm. Data from [15].

of mitochondrial membrane potential, which ultimately leads to apoptosis [55]. Our studies indicate that HupA can improve mitochondrial function in Aβ-exposed PC12 cells by reducing the levels of ROS and increasing activities of key components of the respiratory chain and TCA cycle [56]. Thus, HupA may attenuate neuronal apoptosis and aid in the treatment of neurodegenerative disease such as AD.

Fig. 5. Effects of HupA on apoptotic related protein expression in the cortex of rats. (A–C) Vehicle treated group; (D–F) Aβ1–40–treated rats; (G–I) rats treated with Aβ1–40 and daily administration of HupA (0.2 mg/kg, i.p.) for 12 consecutive days; (A, D and G) the Bcl-2 expression; (B, E and H) the Bax expression; (C, F and I) the P53 expression. Scale bar = 20 μm. Data from [15].
Recently, a rat model of transient focal ischemia (middle cerebral artery occlusion, MCAO), which mimics stroke and seriously impairs mitochondrial functions, was used to evaluate protection afforded by HupA. In this model, we further demonstrated the protective effect of HupA against mitochondrial dysfunction. MCAO followed by reperfusion caused significant neurological deficits and brain infarction in rats, severely impaired activities of mitochondrial respiratory chain enzymes (complex I, complex II–III and complex IV) and α-KGDHC, increased the production of ROS, and induced mitochondrial swelling. Those injuries were significantly attenuated by HupA treatment [57].

In addition, a number of studies have shown that the peripheral anionic site of AChE might contribute to Aβ pathology [58–61], which is of great importance to the neurotoxicity of Aβ. However, this site is not apparently perturbed by HupA, although the X-ray crystal graph showed that HupA interfered with the hydrogen bonding networks formed by Tyr70, Asp72 and Tyr121 (located in the peripheral anionic site) [62]. Further studies will be needed to clarify the precise mechanisms involved in the protection of HupA on Aβ pathology.

2. Effects of huperzine A on inflammation

Inflammatory mechanisms have been strongly linked to the pathogenesis of both AD and VaD [63,64]. It is reported that inflammatory cytokines, such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6), located close to amyloid plaques [65], which might be cytotoxic when chronically produced [66] and might stimulate the production of Aβ peptides [67]. High levels of TNF-α and IL-6 have been reported in the cerebrospinal fluid of patients with VaD [68,69], which suggested a possible involvement of inflammatory mechanisms in the pathogenesis of cognitive impairment in patients with cerebrovascular disease.

In our recent study, we proved the protective effects of HupA against focal cerebral ischemia injury, and suggested that this anti-ischemia effect might associate with anti-inflammatory effect. In the in vivo model, HupA could significantly restore regional cerebral blood flow, reduce infarct area, decrease over-expression of proinflammatory factors including TNF-α and IL-1β in both ipsilateral cortex and striatum, and suppress activation of glial cells in ischemic penumbra (unpublished data). Similarly, the anti-inflammatory effects of HupA was also proven in oxygen–glucose deprivation (OGD)-induced in vitro model of ischemia in C6 gliala cells [70]. OGD followed by reperfusion caused significant phosphorylation and degradation of iκB and nuclear translocation of nuclear factor-kappa B (NFκB), triggered over-expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and nitric oxide (NO) in C6 cells. Along with inhibiting AChE activity, treatment with HupA inhibited activation of NFκB, attenuated iNOS, COX-2 and NO over-expression, and promoted survival in C6 cells subjected to OGD insult.

2.1. Other neuroprotective effects of HupA

HupA not only affect the level of ACh in brain, it could also target on noradrenergic (NE) and dopaminergic (DA) system. NE, DA and ACh levels were significantly increased after administration of HupA in medial prefrontal cortex (mPFC) of rats with Aβ injection into bilateral NBM. On the contrary, neither Aβ nor HupA had effects on 5-hydroxytryptamine (5-HT) concentration in mPFC [71], which indicated that the neuroprotective effects of HupA on Aβ toxicity might be helpful in recovering the ACh, DA and NE levels in AD or other neurodegenerative diseases with abnormalities in these neurotransmitters. Moreover, HupA was also found to reduce glutamate-induced calcium mobilization, but did not affect elevations in intraneuronal free Ca2+ caused by KCl or (−)Bay K 8644 [72]. Further study showed that HupA non-competitively inhibited [3H]MK-801 and [3H]TCP binding, it could also dose-dependently inhibited the NMDA-induced toxicity [73]. Recent study showed that HupA was unlikely to interact with the glutamate or glycine binding site of the NMDA receptors. In contrast, spermidine could dose-dependently attenuate the inhibitory effect of HupA on NMDA receptor [74]. Therefore, HupA is a non-competitive antagonist of the NMDA receptors, acting at one of the polyamine binding sites. Since glutamate-induced excitotoxicity has been implicated in the pathogenesis of AD, the NMDA antagonist activity together with the AChE inhibition of HupA might slow down the progress of AD and improve the symptoms, it probably can be an alternative to the new strategy in AD therapy with the combination of donepezil and memantine. The antagonist effect on NMDA receptor in rat cerebral cortex was also found in other AChEIs including donepezil, galantamine, physostigmine, and this effect seems not to be related with their AChE inhibitory efficiency [75].

Different from other AChE inhibitors such as physostigmine and galanthamine, which are known to be allosteric potentiating ligands of nicotinic acetylcholine receptors (nAChRs) and has been proposed for a new strategy for the treatment of AD [76,77], HupA had no direct effect on the amplitude or kinetics of nAChRs [78]. The involvement of HupA in different neurotransmission systems might not due to effects on receptors levels, since HupA was shown to have no effects on the level of both subtypes M1 and M2 muscarinic acetylcholine receptors, nAChR and glutamate receptor, it only had a minimal inhibition of α-1 adrenergic receptor and benzodiazepine receptor [73].

3. Conclusion

AD and VaD are two complicated brain diseases, which likely involve multiple mechanisms. Therefore, single target drug might exert limited clinical effects. In fact, a drug with multiple molecular targets or combined usage of two or more drugs has attracted intense research as a promising therapeutic strategy for these syndromes. Interestingly, studies indicate that natural herbal medicine—HupA, besides its well proven effects of enhancing cholinergic function, possesses other neuroprotective effects such as regulating APP metabolism, alleviating Aβ-induced oxidative stress, apoptosis and mitochondrial
failure, and anti-inflammation. Therefore, it might not only contribute to symptomatic improvement, but also slow down the progression of AD and VaD by protecting the brain cells from external insults and enhancing self-defensive system. The studies of HupA could provide useful clues for developing more potent and comprehensive therapeutic strategies for AD and VaD. However, further study will be needed to understand the precise molecular mechanisms.

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References

amyloid generation in human embryonic kidney 293 APP Swedish A


