Huperzine B protects rat pheochromocytoma cells against oxygen-glucose deprivation-induced injury

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ABSTRACT

AIM: To test the ability of huperzine B (HupB) to alleviate injury from oxygen-glucose deprivation (OGD) in the rat pheochromocytoma line PC12 cells. METHODS: After OGD for 3 h and reoxygenation for 24 h, neuronal morphology was observed by phase-contrast microscopy; cell survival was quantified by the reduction of MTT; malondialdehyde (MDA) was determined by the thiobarbituric acid; superoxide dismutase (SOD) was assayed by a modification of the xanthine/xanthine oxidase; and lactate (LA) was measured according to Marbach and Weil. RESULTS: OGD for 3 h and reoxygenation for 24 h triggered death in nearly 70 % of cells, along with major changes in morphology and biochemistry including elevated level of MDA, SOD activity, and LA content. Cells pretreated with HupB 1-100 µmol/L for 2 h showed significantly improved survival and reduced biochemical and morphologic signs of toxicity. CONCLUSION: HupB protected PC12 cells against OGD-induced injury, most likely by alleviating disturbances of oxidative and energy metabolism.

INTRODUCTION

Alzheimer’s disease (AD) and vascular dementia (VD) are the most common dementing disorders, accounting for 90%-95 % of all dementias[1]. Vascular risk factors are normally associated with cerebrovascular disease, which may lead to VD. However, more and more recent studies suggest that cerebral ischemia may be involved in AD as well as dementia in general[2]. Based on the “cholinergic hypothesis,” cholinesterase inhibitors (ChEIs) have been developed as promising agents for palliative therapy of AD[3]. Studies on pathogenic mechanisms have revealed that patients with VD exhibit cholinergic abnormalities and disturbance of cognitive function similar to those in AD[4]. It is therefore worth entertaining the possibility that certain types of ChEIs might be able to alleviate cerebral ischemic injury. Several evidences have proved that some ChEIs could protect brain against ischemia[5,6]. In a previous study, we have demonstrated that huperzine A (HupA) was shown to protect rat pheochromocytoma cells against oxygen-glucose deprivation (OGD)[7]. Huperzine B (HupB), a novel Lycopodium alkaloid isolated from the Chinese herb Huperzia serrata (Fig 1), would be a promising candidate in AD and VD therapy, due to a number of advantages over tacrine,
such as highly selective inhibition on brain acetylcholinesterase (AChE), and lower peripheral cholinergic side effects\(^\text{[8,9]}\). The chemical structure has some similarity between HupA and HupB, it will be, therefore, interesting to know whether HupB has a similar effect with HupA on OGD-induced injury. The present investigation was designed to examine the neuroprotective effects of HupB against OGD-induced injury in the rat pheochromocytoma cell line PC12.

**MATERIALS AND METHODS**

**Materials** HupB, colorless powder with purity > 98 %, was prepared by Department of Phytochemistry in this institute, and dissolved with phosphate buffered saline (PBS). The reagents used in this experiment are as follows: Dulbecco’s modified Eagles medium (DMEM, Gibco) supplemented with 10 % heat-inactivated newborn calf serum (NCS), penicillin 1×10^5 U/L, and streptomycin 100 mg/L; Malondialdehyde (MDA), superoxide dismutase (SOD), and lactate (LA) Assay kits (Nanjing Jiancheng Bio-Tek Corporation); Glucose-free Earle’s balanced salt solution (EBSS, pH 7.4); Coomassie brilliant blue (Fluka Chemie); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma).

**Cell cultures and oxygen-glucose deprivation**

PC12 cells were high passages from the American Type Culture Collection (ATCC) and maintained at 37 °C in a humidified atmosphere containing 5 % CO\(_2\). Cells were seeded at a density of 1.5×10^5/L in DMEM, supplemented with 10 % heat-inactivated NCS, penicillin 1×10^5 U/L, and streptomycin 100 mg/L. Experiments were carried out 24 h after cells were seeded. For OGD insult, the original media were removed and the cells were washed with a glucose-free Earle’s balanced salt solution. Then the cultures were placed in fresh glucose-free EBSS and held in an incubator containing 95 % (v/v) N\(_2\) and 5 % (v/v) CO\(_2\) at 37 °C for 3 h. At the end of the exposure period, glucose was added, and the cells were returned to 5 % CO\(_2\) and 95 % room air at 37 °C for an additional 24 h. HupB 0.01-100 µmol/L was added into the cultures 2 h before OGD treatment. Control cultures were maintained in the incubator under normal conditions.

**Cell survival determination** Cell survival was evaluated by the ability to reduce MTT, an indication of metabolic activity. This viability assay was conducted in 96-well plates and read by spectrophotometric measurement with a microplate reader (Bio-Tek Instruments, USA). Morphological observation was also used to evaluate cell injury with a phase-contrast microscope.

**Biochemical examinations** The cultures were washed with ice-cold PBS, pooled in 0.1 mol/L PBS-0.05 mmol/L edetic acid-0.5 % Triton X-100 buffered solution, and homogenized. Homogenates were centrifuged at 1000×g at 4 °C for 30 min, and supernatants were saved. MDA, a compound produced during lipid peroxidation, was determined by the thiobarbituric acid method\(^\text{[10]}\). SOD was assayed by a modification of the xanthine/xanthine oxidase method\(^\text{[11]}\). LA was measured according to Marbach and Weil\(^\text{[12]}\). Protein was measured by Coomassie blue protein-binding using bovine serum albumin as standard.

**Statistical analysis** Experiments were performed in triplicate, and results were expressed as mean±SD.
Data were evaluated for significance with one-way ANOVA followed by Tukey’s test, using SPSS, a computerized statistical package.

RESULTS

Morphological observation After OGD for 3 h and reoxygenation for 24 h, most cells lost their neurites and assumed a rounded shape and some were lysed or replaced by debris. In contrast, cells in cultures pre-treated with HupB at concentrations of 1-100 µmol/L for 2 h appeared much better preserved. Furthermore, cells in cultures maintained under standard condition treated with HupB 100 µmol/L for 29 h showed no significant difference compared with control PC12 cells (Fig 2).

Cell survival determination As determined by MTT reduction, PC12 cells were very sensitive to OGD insult. OGD for 3 h and reoxygenation for 24 h induced death in nearly 70% of cells (P<0.01 vs control group). HupB at concentration higher than 1 µmol/L increased significantly the cell survival (P<0.05 vs OGD group), and attenuated cell injury to modest but equivalent extents (Fig 3). Moreover, HupB 0.01-100 µmol/L had no direct effect on the viability of PC12 cells maintained under normal condition (P>0.05 vs control group) (Fig 4).

Biochemical examination In the present experiment, OGD for 3 h and reoxygenation for 24 h caused a nearly 180% increase in level of MDA, the marker of lipid peroxidation, and an about 40% increase in SOD activity and LA content, respectively. Pretreatment with HupB 1-100 µmol/L for 2 h attenuated the increase in level of MDA (Fig 5), SOD activity (Fig 6), and LA content (Fig 7). HupB at a concentration of 100 µmol/L had no direct effect on MDA level, SOD activity, and LA content in PC12 cells maintained with normal oxygen and glucose (P>0.05 vs control group).

DISCUSSION

It has been well documented that ischemia can result in dysfunction of oxidative metabolism and reduced energy metabolism, linked to the degeneration of neuron[13]. Our results are consistent with the conclusion that OGD caused cellular damage along with a dysfunction in oxidative and energy metabolism.

Lipid peroxidation is associated with degenerating neurons. Decomposing peroxides liberate carbonyl fragments such as MDA, a highly reactive and cytotoxic species that can be responsible for neuronal death. A previous study in vivo observed elevations of MDA level at early time points, minutes to hours after ischemic

![Fig 2. Effects of huperzine B (HupB) on PC12 cells in morphology. Cells exposed to OGD for 3 h and reoxygenation for 24 h observed with phase-contrast microscope. (A) Control PC12 cells maintained under normal conditions. (B) Control PC12 cells treated with HupB 100 µmol/L for 29 h, cells morphology was not affected. (C) PC12 cells exposed to OGD for 3 h and reoxygenation for 24 h lost neurites demonstrating round shape and some of which were lysed or replaced by debris. (D) PC12 cells pre-incubated for 2 h with HupB 10 µmol/L then exposed to OGD insult, cells preserved. Scale bar=50 µm.](image-url)
We found that MDA level rose significantly as a result of free radical generation induced by OGD. The accompanying rise in SOD activity was presumably a compensatory response to the free radical stress. The precise mechanism of increased SOD activity in OGD is not clear, but one possibility is increased SOD gene expression as a feedback to the accumulation of free radicals. During ischemia, anaerobic glycolysis becomes prominent, leading to the accumulation of LA.

Fig 3. Effects of huperzine B (HupB) on cell survival (MTT reduction) in PC12 cells under oxygen-glucose deprivation for 3 h and reoxygenation for 24 h. HupB at concentrations of 0.01-100 μmol/L was added into the culture for 2 h in advance. At least four independent experiments carried out, each in triplicate. Means±SD. *P<0.01 vs control group. †P<0.05, ‡P<0.01 vs OGD group.

Fig 4. Effects of huperzine B (HupB) on cell survival (MTT reduction) in PC12 cells without OGD treatment. HupB at concentrations of 0.01-100 μmol/L was added into the culture for 29 h. At least four independent experiments carried out, each in triplicate. Means±SD.

Fig 5. Effects of huperzine B (HupB) on lipid peroxidation levels in PC12 cells. Cells under OGD for 3 h and reoxygenation for 24 h. HupB at concentrations of 1-100 μmol/L was added into the culture for 2 h in advance. MDA level in control PC12 cells was (4.1±3.7) nmol/mg protein. At least three independent experiments carried out, each in triplicate. Means±SD. *P<0.01 vs control group. †P<0.05, ‡P<0.01 vs OGD group.

Fig 6. Effects of huperzine B (HupB) on antioxidant enzyme activities in PC12 cells. Cells under OGD for 3 h and reoxygenation for 24 h. HupB at concentrations of 1-100 μmol/L was added into the culture for 2 h in advance. SOD activity in control PC12 cells was (546.4±77.2) unit/mg protein. Statistical comparison was made using one-way ANOVA followed by Tukey's test. At least four independent experiments carried out, each in triplicate. Means±SD. *P<0.01 vs control group. †P<0.05, ‡P<0.01 vs OGD group.
A chemical marker of ischemia in experimental animals and in clinic. Our observation of elevated LA after OGD suggests the disturbed glucose homeostasis. Thus, in several important respects, the OGD model appears appropriate for in vitro evaluation of agents as neuroprotectants in ischemia.

The ability of HupB to diminish overproduction of MDA and LA while enhancing cell survival is evident for at least a partial reversal of the redox disequilibrium caused by OGD. The reduction of SOD activity by HupB may reflect a decreased production of the free radicals being generated from metabolic reactions that are exaggerated in OGD.

Previous studies have proved that the efficacy of ChEIs in protecting against OGD insult might be unrelated to inhibition of acetylcholinesterase, and anticholinesterases may be generally neuroprotective[7]. Such findings raise important questions about the underlying mechanisms. The present results suggest that HupB exerts its protective effects against OGD specifically at the level of this derangement of oxidative and energy metabolism. This conclusion is in accordance with recent studies of HupB against hydrogen peroxide-induced injury in vitro[15]. Therefore, HupB may somehow act upon the free radical system, either directly or indirectly. Other potential mechanisms can also be considered. HupB has been demonstrated to antagonize cerebral NMDA receptor[16]. Moreover, we should not reject a neuroprotective role for cholinesterase inhibition and elevated acetylcholine, at least in vivo, since acetylcholine can potentiate the effects of nerve growth factor whose protective actions against ischemic insult are well-documented[17].

In summary, there are multiple ways in which HupB could protect neurons against OGD, or clinical ischemia. So we propose that HupB is not only a potential agent for AD, but also may be beneficial in VD and other neurodegenerative disorders with an ischemic component.

REFERENCES


