Huperzine A attenuates apoptosis and mitochondria-dependent caspase-3 in rat cortical neurons

Jin Zhou, Xi Can Tang*

State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 294 Tai-yuan Road, Shanghai 200031, PR China

Received 13 May 2002; revised 15 July 2002; accepted 17 July 2002

First published online 26 July 2002

Edited by Vladimir Skulachev

Abstract The neuroprotection of huperzine A against apoptosis was investigated. In cultures of rat primary cortical neurons, neuronal apoptosis was induced by serum deprivation for 24 h, which was accompanied by enhanced caspase-3 activity and the release of cytochrome *c* into the cytosol from mitochondria. Pretreating the neurons for 2 h with huperzine A (0.1–10 μM) improved neuronal survival. Huperzine A at a concentration of 1 μM significantly attenuated apoptosis by inhibiting the mitochondria–caspase pathway directly and indirectly.

Key words: Huperzine A; Serum deprivation; Apoptosis; Caspase; Mitochondrion

1. Introduction

Apoptosis is the process by which neurons die during normal development and also a feature of chronic and acute neurodegenerative diseases and stroke [1]. The family of cysteine proteases known as caspases play a central role in apoptosis, contributing to a signalling cascade between death promoting stimuli and the cleavage of protein substrates which contribute to the characteristic apoptotic morphology [2]. In response to the death stimuli, the mitochondria may then initiate apoptosis through the release of cytochrome *c* and activation of the intrinsic caspase pathway [3]. Huperzine A, a Lycopodium alkaloid isolated from the Chinese herb *Huperzia serrata*, has proved to be one of the most promising agents in the therapy of Alzheimer's disease. Interestingly, previous studies have revealed that huperzine A, in addition to its potent acetylcholinesterase inhibition, also exhibited broad neuroprotection against neurotoxicity induced by glutamate, hydrogen peroxide, β-amyloid peptide fragment 25–35 and oxygen–glucose deprivation [4–7]. Furthermore, it has been shown that this agent has the ability to attenuate apoptosis induced by hydrogen peroxide, β-amyloid peptide fragment 25–35 and oxygen–glucose deprivation by altering the expression of apoptosis-related genes [8–10]. However, the precise mechanism of huperzine A's action against apoptosis in neurons remains to be elucidated. In primary neuronal cultures, serum deprivation can induce a marked neuronal death mainly linked to an apoptotic process revealed by morphological and molecular methods [11,12]. Using this injury paradigm, the present experiments were designed to investigate the effect of huperzine A on neuronal apoptosis, especially on the mitochondria–caspase pathway.

2. Materials and methods

2.1. Neuronal culture and serum deprivation

Rat cortical neurons were prepared from 16–18 day old embryos according to a previously reported procedure [13]. The dissociated cells were seeded in poly-L-lysine coated plates at a density of 5×10^5/cm and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were kept in an atmosphere of 5% CO_2 at 37°C and the fresh medium was changed twice weekly. For serum deprivation, the cells were incubated in serum-free medium (DMEM) for 24 h after 7 days in vitro. At the time of treatment, neurons accounted for >95% of total cells (immunostained with a neurofilament antibody). Huperzine A (colorless powder, purity >98%, provided by the Department of Phytochemistry, this Institute) was dissolved and diluted in phosphate-buffered saline (PBS) and added into the culture 2 h before serum deprivation.

2.2. Cell survival assays

Cell survival was evaluated by the ability to reduce MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma), an indication of metabolic activity [14]. This viability assay was conducted in 96 well plates and read by spectrophotometric measurement with a microplate reader (BioTek Instruments, USA).

2.3. Analysis of apoptosis rate by flow cytometry

After serum deprivation for 24 h, neurons were harvested by centrifugation and washed with PBS for the analysis of the apoptosis rate. Cell pellets were fixed in 70% ethanol at 4°C. After centrifugation at 1200 rpm for 5 min, cells were resuspended in 0.5 ml DNA staining reagent containing 50 μg/ml propidium iodide, 50 μg/ml RNase A, 0.1% Triton X-100 and 0.1 mM EDTA (pH 7.4). The apoptosis rate was analyzed using a Becton Dickson FACStar Plus flow cytometer [15].

2.4. Electrophoresis analysis of DNA fragmentation

Neurons ($\sim1\times10^7$) were lysed in 500 μl lysis buffer (20 mM EDTA; 20 mM Tris–HCl, pH 8.0; 0.5% sodium dodecyl sulfate; 100 μg/ml proteinase K) at 37°C for 12 h, then incubated with 50 μg/ml RNase A at 37°C for an additional hour. DNA in the lysate was extracted with equal volumes of chloroform/isoamyl alcohol, then with chloroform. DNA was precipitated with 2 volumes of ethanol in the presence of 0.3 M sodium acetate. After centrifugation at 15000 rpm for 15 min, the DNA pellets were washed with 70% ethanol, air dried and dissolved in 20 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6). The DNA sample was electrophoresed on 1.5% aga-

*Corresponding author. Fax: (86)-21-6437 0269.
E-mail address: xctang@mail.shcnc.ac.cn (X.C. Tang).

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; AMC, 7-amino-4-methyl coumarin; COX4, cytochrome *c* oxidase subunit IV

0014-5793/02/$22.00
PII: S0014-5793(02)03107-1

rose gel containing 0.5 μg/ml ethidium bromide at 85 V for 1 h and photographed by an Ultra Violet Gel Documentation System.

2.5. Morphological analysis

Cell morphology was routinely assessed by phase contrast microscopic observation. Nuclear morphology was analyzed by Hoechst 33342. Neurons were washed three times with PBS and labeled with 1 μg/ml Hoechst 33342 (Sigma) for 15 min at room temperature. Cells were observed and photographed on a fluorescence microscope with a UV2A filter.

2.6. Caspase-3 cleavage assay

The fluorogenic substrate for caspase-3 (Ac-DEVD-AMC) is labeled with the fluorochrome 7-amino-4-methyl coumarin (AMC). The substrate produces a blue fluorescence that can be detected by exposure to UV light at 360 nm. AMC is released from these substrates upon cleavage by caspase-3. Free AMC produces a yellow-green fluorescence that is monitored by a fluorometer at 460 nm. The amount of yellow-green fluorescence produced upon cleavage is in proportion to the amount of caspase-3 activity presented in the sample. Assays were performed in duplicate, and at least three independent experiments were carried out.

2.7. Cell fractionation and Western blot analysis

The highly enriched mitochondrial fractions from the cytosolic fractions of cells ($\sim 5\times10^7$) were separated using the ApoAlet cell fractionation kit (Clontech, USA). The location and change of cytochrome *c* was determined by standard Western blot procedure and probed with the specific cytochrome *c* antibody. Meanwhile, cytochrome oxidase subunit IV (COX4), a membrane protein in the inner mitochondrial membrane, was used to be a marker for the mitochondrial-enriched fraction.

2.8. Statistical analysis

Experiments were performed in triplicate. The results are expressed as means ± S.D. Data were evaluated for significance with one-way ANOVA followed by Duncan's multiple range testing using a computerized statistical package.

3. Results

Significant neuronal injury and apoptosis were induced by serum deprivation for 24 h. Active mitochondrial dehydrogenase of living cells can cleave MTT to produce formazan, the amount of which directly correlates with the number of metabolically active cells. As determined by MTT reduction, the survival of neurons was markedly decreased by about 65% after serum deprivation. However, pretreatment with huperzine A at concentrations of 0.1–100 μM for 2 h was able to attenuate cell injury to modest but equivalent extents ($P<0.01$) (Fig. 1). Meanwhile, huperzine A at the above concentrations had no direct effect on the viability of control neurons ($P>0.05$) (data not shown).

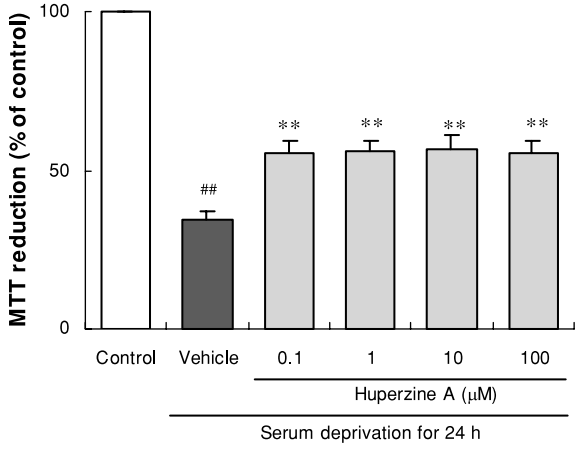

Fig. 1. Effects of huperzine A on cell survival (MTT reduction) in primary cortical neurons. Neurons under serum deprivation for 24 h. Huperzine A at concentrations of 0.1–100 μM added to the culture 2 h in advance. Data expressed as means ± S.D. Statistical comparison was made using ANOVA followed by Duncan's test. At least three independent experiments were carried out, each in triplicate. There was a significant difference between the serum deprivation group and the untreated control group. $^{\#\#}P<0.01$ compared to control group. $^{**}P<0.01$ compared to serum deprivation group.

Neuronal apoptosis was evaluated by different methods. The apoptosis rate was defined as the percentage of cells with subdiploid DNA content (DNA fragmentation) determined with flow cytometry. A significant increase in the apoptosis rate was found when neurons were treated with serum deprivation for 24 h. Pretreatment with 1 μM huperzine A for 2 h significantly blunted the apoptosis rate ($P<0.01$) (Fig. 2). A DNA fragmentation assay was used to determine whether DNA is cleaved into multimers of 180–240 bp associated with endonuclease activity [16]. In our experiments, after serum deprivation, genomic DNA exhibited a 'ladder' pattern on electrophoresis. The same conditions failed to produce an evident DNA laddering when applied after pretreatment with 1 μM huperzine A for 2 h (Fig. 3). Representative morphological changes of neurons with or without serum deprivation are shown in Fig. 4. As observed by phase contrast microscopy, the culture treated with serum deprivation showed signs of neuronal injury including neurite degeneration, shrinkage of cell bodies and accumulation of condensed particles. In Hoechst 33342 staining, serum deprivation triggered the characteristic changes of apoptotic nuclear morphology. The neuronal nuclei appeared smaller and more brightly stained, with evident condensation and fragmentation. In contrast, cells in cultures pre-incubated with huperzine A before serum deprivation appeared remarkably preserved and the above alterations were significantly attenuated.

The assay on caspase-3 activity and the release of cytochrome *c* from mitochondria showed that serum deprivation induced the activation of caspase-3-like protease with an increased cleavage of Ac-DEVD-AMC, the substrate for caspase-3, by about 100%. Pretreatment with huperzine A for 2 h attenuated the substrate cleavage, indicating an inhibition of caspase-3 (Fig. 5A). As shown in Fig. 5B, a 15 kDa band corresponding to cytochrome *c* was observed in the cytosolic fraction after serum deprivation, indicating that apoptosis involves mitochondrial release of cytochrome *c* to the cytosolic fluid. However, huperzine A, pre-incubated in the neuronal culture for 2 h at a concentration of 1 μM, suppressed the release of cytochrome *c*.

4. Discussion

The present study shows that neuronal apoptosis can be activated after serum deprivation for 24 h. With the significant decrease of cell survival by MTT reduction, an increased apoptosis rate was found and genomic DNA exhibited a typical 'ladder' pattern on electrophoresis. These changes were accompanied by condensed or fragmented nuclei characteristic of apoptotic cells. These results are quite consistent with previous findings by Atabay et al. [17]. Apoptosis involves an active, energy-dependent mechanism in which cells participate in their own destruction, which is accomplished by specialized

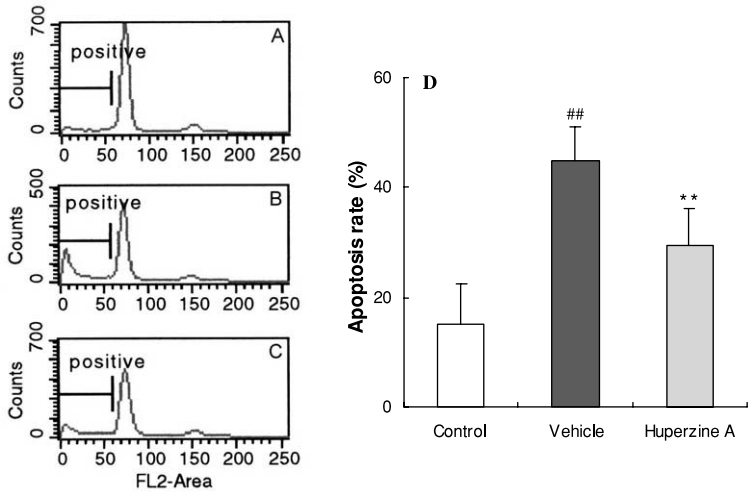

Fig. 2. Determination of apoptosis rate by flow cytometric DNA analysis. A–C: Representative histograms of different groups. A: Control primary cortical neurons. B: Neurons exposed to serum deprivation for 24 h. C: Neurons pre-incubated 2 h with 1 μM huperzine A, then treated with serum deprivation for 24 h. D: Results of quantitative determination of apoptosis rate by flow cytometric analysis. Data are expressed as means ± S.D. $^{\#\#}P < 0.01$ compared to control group. $^{**}P < 0.01$ compared to serum deprivation group.

cellular machinery. The central component of this machinery is a proteolytic system involving a family of proteases called caspases. These enzymes participate in a cascade that is triggered in response to proapoptotic signals and culminates in the cleavage of a set of proteins, resulting in disassembly of the cell [18]. After serum deprivation for 24 h, we found that mitochondria released cytochrome *c* to the cytosol, and increased caspase-3 activity, indicating that in response to the death stimuli, the mitochondria may initiate apoptosis through release of cytochrome *c* and activation of the intrinsic caspase pathway. Mitochondria are pivotal in controlling life and death. During apoptosis, cytochrome *c* is released from mitochondria to cytosol. Cytosolic cytochrome *c* is required for the formation of the apoptosome, which is composed of cytochrome *c*, Apaf-1 and procaspase-9 [19]. As a result, caspase-9 is cleaved and activated, which then can process and cleave other caspases, including caspase-3, to orchestrate the biochemical execution of cells. Activation of caspase-3 appears to be a key event in apoptosis. Numerous cellular substrates for caspase-3 have been identified, including poly-(ADP-ribose) polymerase, DNA protein kinase, laminin B, and DNA fragmentation factor. The proteolytic cleavage of these important proteins is directly responsible for the char-

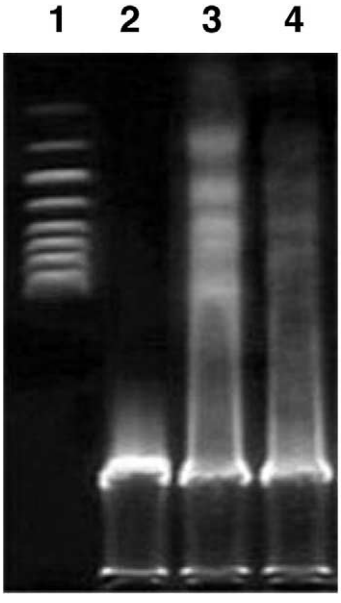

Fig. 3. Effects of huperzine A on electrophoresis pattern of DNA fragmentation in primary cortical neurons induced by serum deprivation for 24 h. Lane 1: DNA marker; lane 2: control culture; lane 3: culture exposed to serum deprivation for 24 h; lane 4: culture pre-incubated 2 h with 1 μM huperzine A and then treated with serum deprivation for 24 h.

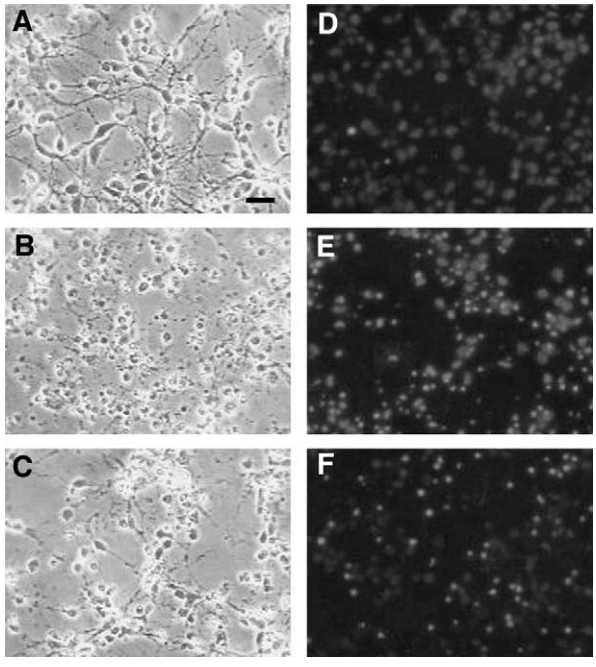

Fig. 4. Effects of huperzine A on primary cortical neurons in morphology. A–C: Observed with phase contrast microscope. D–F: Nuclear morphology of apoptotic cells shown by Hoechst 33342 staining. A, D: Control neurons. B, E: Neurons exposed to serum deprivation for 2 h. C, F: Neurons pre-incubated 2 h with huperzine A 1 μM and then treated with serum deprivation for 24 h. Scale bar = 50 μm.

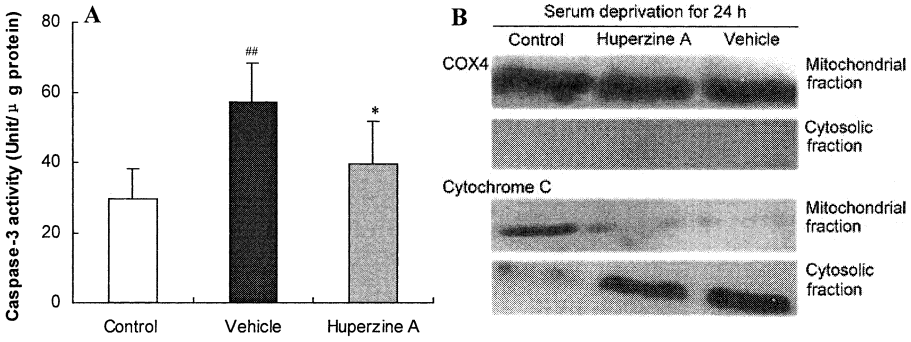

Fig. 5. Effect of huperzine A on caspase-3 activity (A) and Western blot detection of cytochrome *c* and COX4 (B) in primary cortical neurons. Neurons under serum deprivation for 24 h. Huperzine A at a concentration of 1 μM added to the culture 2 h in advance. A: Data expressed as means ± S.D. Statistical comparison was made using ANOVA followed by Duncan's test. At least three independent experiments were carried out, each in triplicate. There was a significant difference between the serum deprivation group and the untreated control group. $^{\#\#}P < 0.01$ compared to control group. $^{*}P < 0.05$ compared to serum deprivation group. B: The mitochondrial and cytosolic fractions were isolated using an ApoAlert cell fractionation kit. They were then processed using the standard Western blot procedure on 12% SDS–PAGE and probe with COX4 antibody (~17 kDa) or cytochrome *c* antibody (~15 kDa). The presence of cytochrome *c* in the cytosolic fraction after induction indicates that apoptosis involves mitochondrial release of cytochrome *c* to the cytosol.

acteristic changes associated with apoptosis, including the cessation of DNA repair, chromatin condensation and internucleosomal DNA fragmentation [20,21].

Huperzine A has been found to exhibit broad neuroprotection in vivo and in vitro [4–10]. A previous study in our laboratory has demonstrated that the ability of huperzine A to attenuate apoptosis induced by oxygen–glucose deprivation may result from its ability to alter the expression of apoptosis-related genes; huperzine A could effectively antagonize the up-regulation of c-jun and bax and the down-regulation of bcl-2 [10]. The present results have confirmed and extended this observation. When neurons were pre-incubated with huperzine A at a concentration of 1 μM, a marked neuroprotection against serum deprivation was induced. Huperzine A significantly attenuated neuronal apoptosis, and inhibited caspase-3 activity and mitochondrial release of cytochrome *c* to the cytosol. Our results suggest that huperzine A might block apoptosis by antagonizing the mitochondrial-dependent caspase pathway directly or indirectly. Combining these observations with the results of huperzine A on apoptosis-related genes, it is proposed that the effects of huperzine A on intrinsic caspase-3 pathway might be a downstream event of regulation of the expression of bcl-2 family genes. Functionally, bcl-2 is a potent cell death suppressor, whose over-expression can prevent cell death in response to a variety of stimuli, including serum and growth factor deprivation. However, bax is a death promoting factor, whose translocation to the mitochondrial membrane might lead to loss of mitochondrial membrane potential and an increase of mitochondrial permeability. Increased mitochondrial permeability results in the egress of cytochrome *c* from the mitochondria and the following activation of caspase-3 [22]. Huperzine A could effectively antagonize the up-regulation of bax and the down-regulation of bcl-2, which might contribute to its effects on mitochondria-dependent caspase pathway. Thus, direct effects of huperzine A on cytochrome *c* and caspase-3 could not be excluded.

There is mounting evidence supporting the notion that huperzine A could be a useful neuroprotective agent. Especially, it has been found that huperzine A exhibits neuroprotection against ischemic injury in vivo and in vitro [7,10,23,24]. Furthermore, the results in this study have provided evidence for the molecular mechanisms of huperzine A against neuronal apoptosis induced by serum deprivation. These findings indicate that, besides Alzheimer's disease, huperzine A might be beneficial in other neurodegenerative diseases, including ischemia-related neurodegenerative diseases.

Acknowledgements: This work was supported by research grants from the Ministry of Science and Technology of China (G1998051110) and the National Natural Science Foundation of China (3001161954, 30123005). The authors are grateful to Prof. I. Hanin for his English revision and to Miss Zhi Fei Wang for her technical help.

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