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Huperzine A protects SHSY5Y neuroblastoma cells against oxidative stress damage via nerve growth factor production

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Abstract

Our previous study demonstrated that huperzine A, a selective acetylcholinesterase inhibitor, stimulates the synthesis of nerve growth factor (NGF) in cultured rat cortical astrocytes. The present studies are designed to examine if huperzine A exerts its neuroprotective activity against oxidative stress damage through increasing the synthesis of NGF in SHSY5Y neuroblastoma cells. Transient exposure of the cells to 200 μM H_2O_2 triggered a significant reduction of cell viability and decreased the mRNA and protein levels of NGF, neurotrophin receptor P75 ($P75^{NTR}$) receptor and tyrosine kinase A (TrkA) receptor. Incubation of cells with 10 μM huperzine A prior to H_2O_2 exposure significantly elevated their survival and restored the mRNA and protein levels of NGF, $P75^{NTR}$ receptor and TrkA receptor. These neuroprotective effects of huperzine A on H_2O_2-induced cytotoxicity were blocked by the TrkA receptor phosphorylation inhibitor K252α, and were antagonized by the mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) inhibitor, PD98059. The present results indicate that the cytoprotective effect of huperzine A is mediated at least partly by up-regulated NGF and NGF receptors. The results also show that the MAP/ERK kinase signal pathway is crucial for huperzine A to protect against H_2O_2-induced damage in SHSY5Y cells.

Keywords: Huperzine A; Oxidative stress; Nerve growth factor; MAP (mitogen-activated protein) kinase; TrkA (tyrosine kinase A) receptor

1. Introduction

Alzheimer's disease is a multifaceted neurodegenerative disorder characterized by loss of brain neurons and progressive deterioration in cognitive, language, executive and behavioral functions. Although cause and mechanism are not very clear, multiple lines of evidence demonstrate that oxidative stress is an early event in the pathogenesis of Alzheimer's disease (Nunomura et al., 2001; Perry and Smith, 1998). Oxidative stress in neurons can cause DNA damage, oxidation of proteins, peroxidation of lipids, and formation of advanced glycosylation end products. This stress can lead to rapid cell death. Therefore, molecules that function as antioxidants or promote cellular antioxidant enzyme activity could be useful in the treatment of Alzheimer's disease. Nerve growth factor (NGF), a target-derived neurotrophic factor, plays an important role in the survival and maintenance of cholinergic neurons in the central nervous system. It has been reported that treatment with NGF restores catalase activity and increases superoxide dismutase (SOD) and GSH peroxidase activity in different brain areas in aged rats (Nistico et al., 1992) and in PC12 cells after oxidative stress damage (Jackson et al., 1990a,b). Thus, NGF may be important in antioxidant defenses in the nervous system.

On binding to tyrosine kinase A (TrkA) receptor, NGF activates several downstream intracellular pathways, including mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase and phosphoinositide-3 (PI-3) kinase. The MAP/ERK signaling pathway plays a pivotal role in suppressing apoptosis (Xia et al., 1995; Yujiri et al., 1998) and PI-3 kinase pathway has a

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role in cell survival (Jackson et al., 1996; Bartlett et al., 1997). These two pathways may be involved in NGF-induced neuroprotection.

Huperzine A, a novel *Lycopodium* alkaloid isolated from the Chinese folk medicine *Huperzia serrata*, is a reversible and selective inhibitor of acetylcholinesterase and has been used in clinical treatment of Alzheimer's disease (Xu et al., 1995). Besides inhibiting acetylcholinesterase, huperzine A can attenuate oxidative injury by multiple modes of action (Wang and Tang, 2005), which include the stimulation of antioxidant enzymes (Xiao et al., 1999) and changes in the expression of apoptosis-related genes (Wang et al., 2001). We recently found that huperzine A increases NGF production in cultured astrocytes (Tang et al., 2005), raising the possibility that similar phenomena contribute to the antioxidative activity of huperzine A. Here we report effects of huperzine A on the expression and synthesis of NGF and its receptors, TrkA receptor and $P75^{NTR}$ receptor, in the context of damage induced by H_2O_2 oxidative stress in SHSY5Y cells. The present findings indicate that increased NGF production may partly explain the neuroprotective effect of huperzine A, which appears to require activity from MAP/ERK kinase in the NGF signaling pathway.

2. Materials and methods

2.1. Drug preparation

Huperzine A, provided by the Department of Phytochemistry at this Institute, is a colorless powder with m.p. 230 °C, and purity >99%. It was dissolved and diluted in phosphate-buffered saline (PBS).

2.2. Cell culture

SHSY5Y neuroblastoma cells, high passages from the ATCC, were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were seeded into plates or dishes in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10%(v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Experiments were carried out 24–48 h after the cells were seeded. Sixteen hours before treatment, the cells were washed with D-Hanks and further incubated in fetal bovine serum-free DMEM containing neuroblastoma growth supplement N2. In order to produce oxidative stress, H_2O_2 was freshly prepared from 30% stock solution prior to each experiment. Preincubation with huperzine A was conducted 2 h before H_2O_2 addition. Assays for cell viability, genes expression and protein level of NGF and NGF receptors were performed at different time after H_2O_2 exposure.

2.3. Assay for cell survival

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. Briefly, SHSY5Y cells were seeded into 96-well plates at a density of 10^4 cells/well, 24 h before H_2O_2 exposure. After a 24 h incubation in 200 μM H_2O_2, MTT solution in phosphate-buffered saline (PBS) was added in a final concentration of 0.5 mg/ml and the incubation continued for 4 h. Finally, an equal volume of a solution containing 50% dimethylformamide and 20% sodium dodecyl sulfate (SDS) (pH 4.8) was added. The mixtures were kept overnight and then the amount of MTT formazan was qualified by determining the absorbance at 570 and 630 nm using a Universal Microplate Reader (Bio-Tek).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

SHSY5Y cells were collected at 2 and 4 h after incubation with 200 μM H_2O_2. Total cellular RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacture's protocols and was quantified by absorbance at 260 nm. RNA purity was determined using the A260/A280 ratio (average >1.85). Total RNA of each sample was first reverse-transcribed into cDNA using Reverse Transcription System (Promega). PCR amplification was performed with reagents from Promega. The cDNA solution was amplified with primers based on the human NGF, TrkA receptor and $P75^{NTR}$ receptor sequences. Primer sequences were: NGF: 5′-CTTCAGCATTCCCTTGACAC-3′ (upstream), 5′-AGCCTTCCTGCTGAGCACACA-3′ (downstream); TrkA receptor: 5′-CCATCGTGAAGAGTGGTCTC-3′ (upstream), 5′-GGTGACATTGGCCAGGGTCA-3′ (downstream); $P75^{NTR}$ receptor: 5′-AGCCAACCAGACCGTGTGTG-3′ (upstream), 5′-TTGCAGCTGTTCCACCTCTT-3′ (downstream); β-actin: 5′-CCTGCGTCTGGACCTGGCTG-3′ (upstream), 5′-CTCAGGAGGAGCAATGATCT-3′ (downstream). Amplification was performed as follows: NGF: 24 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s; TrkA receptor: 40 cycles of 94 °C for 60 s, 55 °C for 30 s and 72 °C for 60 s; $P75^{NTR}$ receptor: 25 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 90 s; β-actin: 30 cycles of 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 60 s. The PCR products were normalized in relation to standards of β-actin mRNA.

2.5. Measurement of NGF

Released NGF was measured in the supernatants by a two-site enzyme-linked immunosorbent assay (ELISA) using the NGF $E_{max}^{®}$ Immunoassay system. Briefly, plates were coated with goat anti-NGF polyclonal antibodies (1:6250) overnight at 4 °C. Non-specific binding sites were saturated with Block and Sample 1 × Buffer. The samples and a standard solution (0–500 pg/ml of NGF) were added and the plates were incubated for 6 h at room temperature. After washing three times, rat anti-NGF monoclonal antibodies (1:4000) were added and incubated overnight at 4 °C. Then after washing five times, horseradish peroxidase (HRP)-conjugated anti-rat IgG (1:100) was added for 2.5 h at room temperature. After washing five times more, TMB was added for a 10 min incubation at room temperature. Reactions were then stopped with 1 N HCl, and absorbance at 450 nm was measured in a Microplate Reader.

2.6. Immunocytochemistry

Cultures were fixed on the cover slides with 10% formaldehyde, 24 h after incubation with H_2O_2. Slides were rinsed with PBST and then blocked with 10% rabbit or goat serum

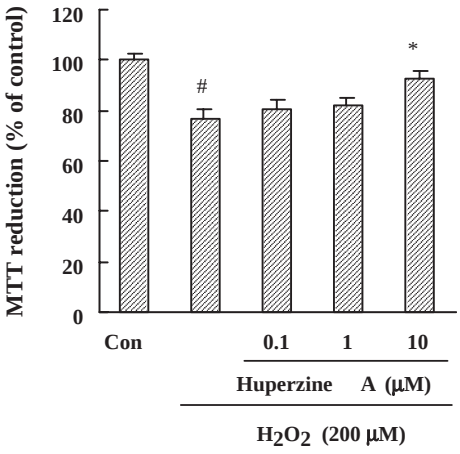

Fig. 1. Attenuation of H_2O_2-induced SHSY5Y cell damage by huperzine A. Cells were incubated with 200 μM H_2O_2 for 24 h. Huperzine A was added to the culture 2 h prior to H_2O_2 addition. Cell viability was assessed by measuring the MTT reduction. Values are the mean ± S.E.M. expressed as a percent of control value. Three separate experiments were performed. $^{\#}P<0.05$ vs. control. $^{*}P<0.05$ vs. H_2O_2-treated group.

(Gibco) in PBST for 2 h at room temperature. Cells were then reacted with anti-TrkA receptor (1 : 500 dilution, Santa Cruz) or anti- $P75^{NTR}$ receptor antibody (1:1000 dilution, Santa Cruz) at 4 °C overnight. Next day, the cells were treated with rhodamine-conjugated goat anti-rabbit secondary antibody (to detect TrkA receptor) or FITC-conjugated rabbit anti-goat secondary antibody (to detect $P75^{NTR}$ receptor). The slides were then washed with PBS and examined under a Nikon fluorescent microscope (ECLIPSE TE2000-E).

2.7. Western blot analysis of TrkA receptor and P75NTR receptor

Cells (1×10^5/ml) harvested 24 h after incubation with H_2O_2 were lysed in 1 × SDS PAGE (polyacrylamide gel electrophoresis) gel loading buffer (Tris–Cl 50 mmol/L PH 6.8, DTT 100 mmol/l, 2%(w/v) SDS, 10%(w/v) glycerol, 0.1% Bromophenol Blue) and boiled in a water bath for 10 min. Protein concentration was determined by Coomassie blue binding method using bovine serum albumin as standard (Bradford, 1976). Equal amounts of protein (40 μg) were then run in each lane of an electrophoresis gel and the separated bands were transferred to a nitrocellulose membrane. After blocking with TBST (Tris buffered saline with

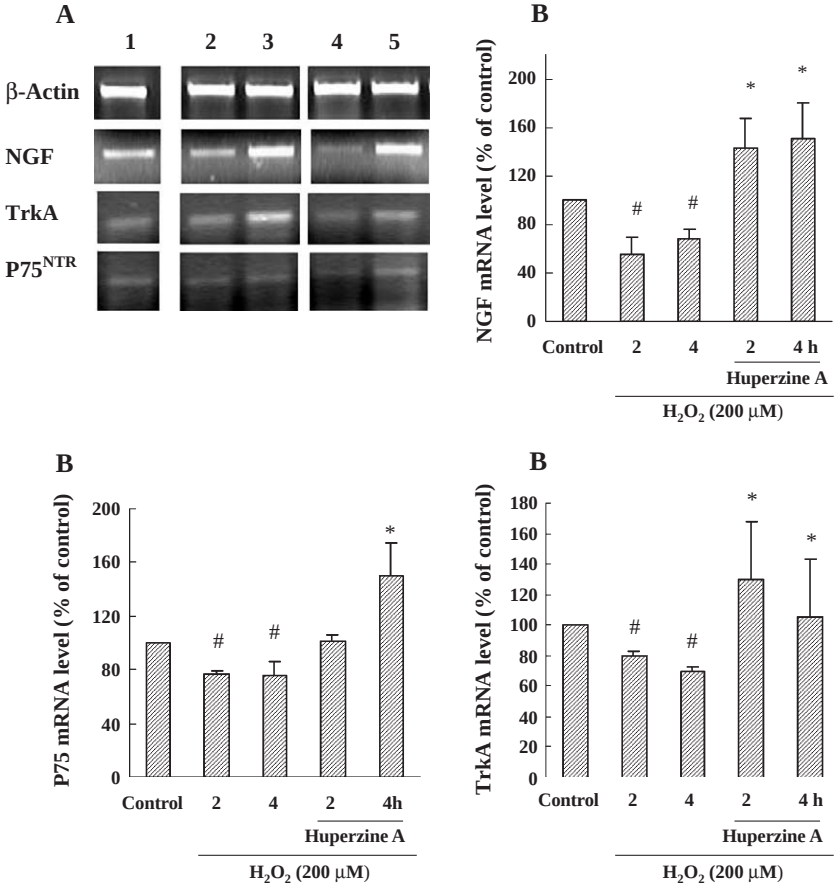

Fig. 2. Effects of huperzine A on H_2O_2-induced expression of NGF, TrkA receptor and $P75^{NTR}$ receptor in SHSY5Y cells by RT-PCR. Cells were exposed to 200 μM H_2O_2 for 2 and 4 h, total RNA was extracted and then subjected to RT-PCR. Preincubation with huperzine A (10 μM) was conducted 2 h before the H_2O_2 was added. (A) The PCR products were normalized by β-actin mRNA. Lane 1, non-treated control at 2 h; Lane 2, 2 h after H_2O_2 treatment; Lane 3, 2 h after treatment of H_2O_2 plus 10 μM huperzine A; Lane 4, 4 h after H_2O_2 treatment; Lane 5, 4 h after treatment of H_2O_2 plus 10 μM huperzine A. (B) Quantitative summary of results in (A). Values are the mean ± S.E.M. expressed as a percent of control value. Three separate experiments were performed. $^{\#}P<0.05$ vs. control. $^{*}P<0.05$ vs. H_2O_2-treated group.

Tween) containing 5% non-fat milk, the membrane was incubated overnight at 4 °C with primary antibodies to TrkA receptor (1:500 dilution, Santa Cruz) and $P75^{NTR}$ receptor (1:1000 dilution, Santa Cruz), followed by HRP-conjugated anti-rabbit or anti-goat IgG at 37 °C for 2 h. Target protein bands were then detected by the enhanced chemiluminescence (ECL) (Pierce) and Kodak film.

2.8. Pharmacological inhibition

After the SHSY5Y cells were seeded into 96-well plates as described above, 100 nM K252α, 100 μM PD98059 or 100 nM wortmannin was added to the cultures (Tsang and Kamei, 2004), which were further incubated for 1 h before the addition of huperzine A. Finally, after 24 h incubation with 200 μM H_2O_2, the percentage of viable cells was determined by MTT assay as described earlier.

2.9. Statistical analysis

Data were expressed as mean ± S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test, with $P<0.05$ as the significant level.

3. Results

3.1. Huperzine A protected the SHSY5Y neuroblastoma against H_2O_2-induced injury

Active mitochondria of living cells can cleave MTT to produce formazan, the amount of which is directly proportional to the living cell number. Consistent with our previous reports

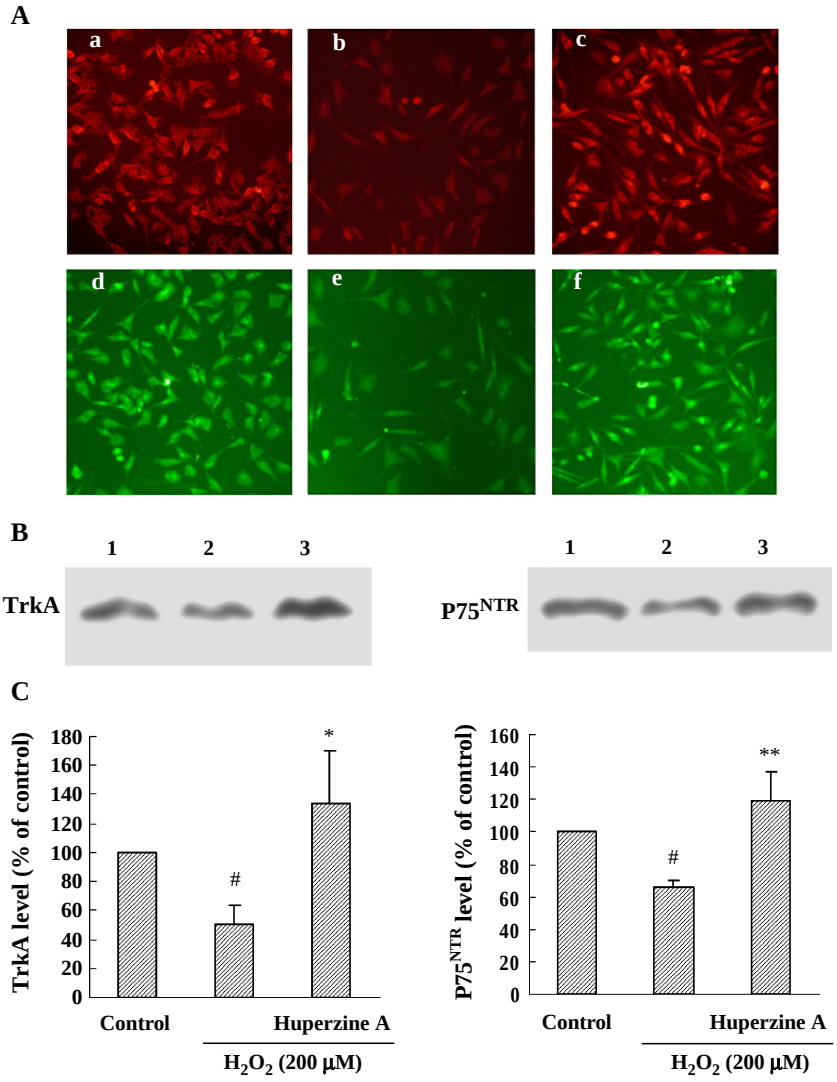

Fig. 3. Effects of huperzine A on TrkA receptor and $P75^{NTR}$ receptor levels in SHSY5Y cells by immunofluorescence assay and Western blot. The tests were performed at 24 h after incubation with H_2O_2. Preincubation with 10 μM huperzine A was conducted 2 h before the 200 μM H_2O_2 was added. (A) immunofluorescence assay results. (a–c) TrkA receptor level; (d–f) $P75^{NTR}$ receptor level. (a, d) control; b,e H_2O_2 treatment; (c, f) huperzine A plus H_2O_2. The figure is representative of three experiments with similar results. (B) Western blot results. (1) control, (2) H_2O_2 group, (3) H_2O_2+huperzine A group. (C) Quantitative summary of results in (B). The data are mean ± S.E.M. expressed as a percentage of control value. $n=5$. $^{\#}P<0.05$ vs. control. $^{*}P<0.05$, $^{**}P<0.01$ vs. H_2O_2-treated group.

(Wang et al., 2001), viability as determined by MTT reduction was decreased about 25% after SHSY5Y cultures were exposed to 200 μM H_2O_2, suggesting that these cells were sensitive to oxidative injury. When the cells were preincubated with 10 μM huperzine A for 2 h, however, H_2O_2-induced toxicity was significantly attenuated (Fig. 1).

3.2. Effects of huperzine A on mRNA and protein levels of NGF, TrkA receptor and $P75^{NTR}$ receptor

For analysis of NGF, TrkA receptor and $P75^{NTR}$ receptor mRNA levels, we used a comparative PCR approach to measure target cDNAs amplified from culture mRNA samples (Fig. 2). Treatment of SHSY5Y cells with 200 μM H_2O_2 induced down-regulation of NGF, TrkA receptor and $P75^{NTR}$ receptor mRNA levels at 2 or 4 h. Cells treated with huperzine A before H_2O_2 exposure resisted these changes. In fact, at both 2 and 4 h after H_2O_2 exposure, the levels of NGF and TrkA receptor mRNA were significantly higher in huperzine A -pretreated cells than in unprotected cells and were also at or above the control level. A similar effect was seen with $P75^{NTR}$ receptor mRNA as well, although it was statistically significant only at 4 h (Fig. 2).

The effects of huperzine A on regulation of these gene products were confirmed by ELISA assay, immunocytochemistry and Western blot. NGF release into culture supernatants was decreased to a very small but significantly degree 24 h after exposure to H_2O_2 (91.3% ±6.4% of control). However, pretreatment with huperzine A prevented this reduction and even enhanced NGF release measurably above the control level (109.3% ±4.2% of control). Immunocytochemistry (Fig. 3A) showed that 24 h treatment with H_2O_2 caused a reduction in cell number along with qualitatively apparent decreases in TrkA receptor and $P75^{NTR}$ receptor immunoreactivity. To quantify the changes in protein levels, Western blot analysis was performed on extracts from the same cultures. This analysis confirmed the immunocytochemical data indicating that NGF receptors were significantly down regulated by H_2O_2 treatment (Fig. 3B, C). Furthermore, the Western blots demonstrated that preincubation with huperzine A preserved the levels of TrkA receptor and $P75^{NTR}$ receptor.

3.3. Effects of K252α, wortmannin and PD98059

We used three pharmacologic agents to determine whether NGF and its downstream pathways contributed to the protection by huperzine A against oxidative cell damage. K252α, an inhibitor of phosphorylation events associated with activation of the TrkA receptor signal transduction pathway, has been reported to block NGF's ability to support neuronal survival. We found that pretreatment with K252α also reduced the survival-supporting activity of huperzine A. In cells treated with H_2O_2 plus K252α, the addition of huperzine A failed to enhance viability (Fig. 4A). Of interest, K252α reduced cell viability on its own, whether H_2O_2 was present or not, probably because it blocked intrinsic signaling from the NGF constitutively secreted by SHSY5Y cells (see Fig. 4A). In any case, the results indicated that NGF and its high affinity receptor do contribute to the protective effects of huperzine A against H_2O_2-induced injury.

Similar experiments also tested the effects of wortmannin and PD98059 on the survival of stressed SHSY5Y cells (Fig. 4B). As measured by changes in the capacity for MTT reduction, the PI-3

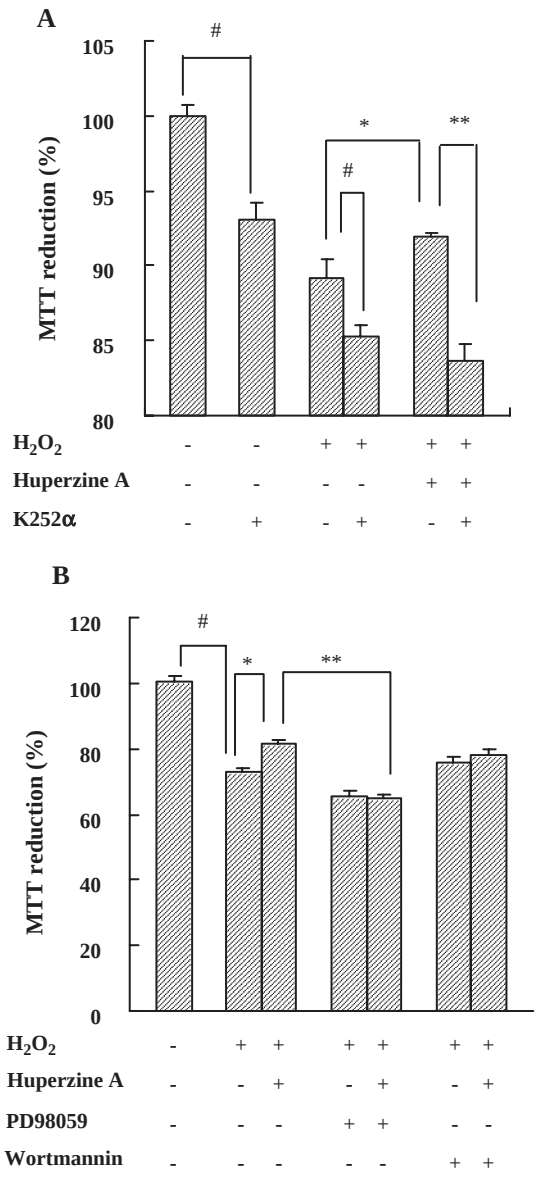

Fig. 4. Effects of K252a, PD98059 and wortmannin on the protection of huperzine A against H_2O_2-induced cell injury in SHSY5Y cells. Cells were pretreated with 100 nM K252a, 100 μM PD98059 or 100 nM wortmannin for 1 h before the addition of 10 μM huperzine A. Huperzine A was conducted 2 h before the 200 μM H_2O_2 was added. After incubation with H_2O_2 for 24 h, cell viability was determined by MTT assay. The data are mean±S.E.M obtained from three independent experiments. (A) Effects of K252a. $^*P<0.05$, H_2O_2 group vs. H_2O_2+ huperzine A group, $^{**}P<0.01$, H_2O_2+huperzine A group vs. H_2O_2+huperzine A+K252a group. $^{\#}P<0.05$, K252a group vs. control, H_2O_2 group vs. H_2O_2+K252a group. (B) Effects of PD98059 and wortmannin. $^*P<0.05$, H_2O_2 group vs. H_2O_2+huperzine A group. $^{**}P<0.01$, H_2O_2+huperzine A group vs. H_2O_2+huperzine A+PD98059 group. $^{\#}P<0.05$ H_2O_2 group vs. control.

kinase inhibitor, wortmannin, did not affect the viability of untreated controls (data not shown) or H_2O_2-treated cells, and it likewise failed to antagonize the protective effect of huperzine A. On the other hand, the MAP/ERK kinase inhibitor PD98059 blocked the beneficial effects of huperzine A even though it did not reduce cell survival on its own (Fig. 4B). Thus, the MAPK/ERK

pathway may be critical in enabling huperzine A to reduce H_2O_2-induced cell damage.

4. Discussion

Neurotrophic factors like NGF not only promote the survival of responsive neurons but also protect them from oxidative injury (Jackson et al., 1990a,b). In vitro, NGF has been shown to enhance resistance to oxidative stress in neuron-like PC12 cells by inducing free radical scavenging enzymes like catalase (Sampath et al., 1994). Our previous studies showed that huperzine A acts in a similar manner to activate the expression of antioxidant enzymes that protect neurons against agents like H_2O_2 (Xiao et al., 1999). We also observed that huperzine A can increase NGF secretion in astrocytes and induce neurotrophin activity in neuron-like PC12 cells (Tang et al., 2005). In light of these findings, it is reasonable to propose that NGF and its downstream signaling system might participate in the neuroprotective effects of huperzine A. Our present study with SHSY5Y cells used H_2O_2 to generate an oxidative stress sufficient to cause cell loss along with substantial decreases in the mRNA and protein levels for NGF, TrkA receptor, and $P75^{NTR}$ receptor. In this setting huperzine A not only reduced the overt signs of cytotoxicity, is also preserved the expression of NGF and its receptors. These results led us to consider that NGF and NGF signaling might be involved in the neuroprotective effects of huperzine A.

NGF mediates its effects through TrkA receptor, $P75^{NTR}$ receptor, and perhaps other receptors (Bothwell, 1995; Kaplan and Miller, 1997). TrkA receptor is a single-pass transmembrane protein that function as a receptor tyrosine kinase and mediates many of the neurotrophic actions classically ascribed to NGF (Loeb et al., 1991). Our data are consistent with the possibility that increased signaling through NGF receptors accounts for a good share of huperzine A's ability to protect neuron-like cells against oxidative damage. The observed increase in NGF expression and the preservation of TrkA receptor should both lead to increased signaling. A key role for such signaling is supported by the observation that the TrkA receptor inhibitor, K252α, blocked the neuroprotective activity of huperzine A.

MAP/ERK lies in the downstream signaling pathway of NGF and plays a pivotal role in suppressing apoptosis and promoting cell survival throughout the nervous system. After binding NGF, TrkA receptor signals through a serious of kinases, including MAP/ERK and MAPK, leading to activation of specific gene programs through effectors such as cAMP regulatory element binding protein, or CREB (Grewal et al., 1999). We found that huperzine A did not protect against H_2O_2 when an inhibitor of MAP/ERK kinase was present. This finding strengthens the view that the antioxidative effects of this compound involve NGF signaling. Taken the evidence together, it is likely that huperzine A rescued NGF expression and TrkA receptor levels and then activated MAP/ERK pathway, resulting in protection of SHSY5Y cells against H_2O_2-induced cytotoxicity.

The PI3K signaling pathway is another downstream pathway involved in cell survival related to TrkA receptor activation. It has been established in several reports that the PI3K pathway promotes cell survival by enhancing the expression of anti-apoptotic proteins and inhibiting the activity of pro-apoptotic ones (Downward, 2004). Our previous work has shown that huperzine A attenuated apoptosis by up-regulating anti-apoptotic protein, Bcl-2, and down-regulating pro-apoptotic proteins, Bax, P53 and caspase-3 (Wang et al., 2001). In this study, however, the PI3K antagonist, wortmannin failed to block the neuroprotective activity of huperzine A. Hence it appears that some anti-apoptotic effects of huperzine A must involve other signaling networks.

In conclusion, the present study demonstrated that NGF and TrkA receptor mediate key events required for the neuroprotective actions of huperzine A. Among the downstream signaling events triggered by NGF action at TrkA receptor, activation of the MAP/ERK kinase pathway may be particularly important for huperzine A's ability to protect against oxidative stress in SHSY5Y cells.

Acknowledgements

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