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Effects of huperzine A on memory deficits and neurotrophic factors production after transient cerebral ischemia and reperfusion in mice

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Abstract

This study is to investigate the effects of huperzine A on memory deficits, neuronal damage and neurotrophic factors production after transient cerebral ischemia and reperfusion in mice, as well as the potential downstream signaling pathway. Bilateral common carotid occlusion (BCCAo) combined with systemic hypotension induced severe memory deficits in a water maze task and neuronal degeneration in cerebral cortex and hippocampus in mice. Oral administration of huperzine A (0.2 mg/kg, once per day, started 2 days before surgery and lasted for 7 days after surgery) markedly attenuated the memory deficits and neuronal damage. Meanwhile, huperzine A significantly increased the mRNA and protein levels of NGF, BDNF and TGF-β_1, and potentiated phosphorylation of MAPK/ERK 1/2 in both cerebral cortex and hippocampus compared with transient cerebral ischemia and reperfusion group. This study provides evidence for the protective effects of huperzine A against transient cerebral ischemia and reperfusion in mice, and suggests potentially important roles that neurotrophic factors might play in these effects. It also indicates that the MAPK/ERK pathway might be involved in the in vivo neurotrophic effects of huperzine A against transient cerebral ischemia and reperfusion.
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Keywords: Huperzine A; Acetylcholinesterase inhibitor; Transient cerebral ischemia and reperfusion; Learning and memory; Nerve growth factor; Brain-derived neurotrophic factor; Transforming growth factor-β_1; Mitogen-activated protein kinase/extracellular signal-regulated kinase

1. Introduction

Brain is highly sensitive to ischemic insults. Ischemia, such as what occurs during acute ischemic stroke, mainly affects regions of the cerebral cortex as well as the hippocampus, which is important for learning and long-term memory. Mechanisms by which neuronal damage occurs involve elevation of intracellular Ca^{2+} levels, overexcitation and generation of free radicals (Choi and Rothman, 1990; Schurr and Rigor, 1992). However, there is no comprehensive pharmacotherapy by far. At present, rescue of damaged neurons and stimulation of neurogenesis are theoretically attractive strategies for the treatment of neurodegenerative diseases.

Several endogenous neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and transforming growth factor-β_1 (TGF-β_1) have been identified and found to be critical for development, differentiation as well as maintenance of distinct populations of neurons. NGF can ameliorate neuronal degeneration in rats subjected to ischemic insults (Shigeno et al., 1991; Pechan et al., 1995). BDNF was reported to significantly reduce the size of infarction and neurological deficits in focal ischemic rats (Schabitz et al., 1997). Additionally, exogenous BDNF administered prior to ischemia has been shown to partially prevent neuronal death in the CA1 area of the hippocampus (Beck et al., 1994). A couple of studies demonstrate that TGF-β_1 has the capacity to reduce the infarct size after focal cerebral ischemia in mice and rabbits (Prehn et al., 1993; Gross et al., 1993), and ameliorate injury in

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CA1 hippocampal neurons caused by transient global ischemia in rats (Henrich-Noack et al., 1996). However, clinical use of these neurotrophic factors is limited by their inability to reach the brain after systemic administration. Therapeutic application of neurotrophic factors therefore necessitates intracranial injections, transplantation of cells secreting neurotrophic factors or gene therapy. Such approaches have produced promising results in several animal models of cerebral ischemia (Shigeno et al., 1991; Pechan et al., 1995; Schabitz et al., 1997; Beck et al., 1994; Prehn et al., 1993; Gross et al., 1993; Henrich-Noack et al., 1996; Yagi et al., 2000). Attempts are being made to discover certain small molecules with the ability to activate or enhance neurotrophic signaling, which might provide an alternative therapeutic approach.

Neurotrophic factors exert their actions by binding to specific transmembrane receptors with intracellular tyrosine kinase domains. Ligand binding induces dimerization of the receptor and activation of the intrinsic tyrosine kinase, leading to phosphorylation of specific tyrosine residues located at the intracellular domain. These events result in recruitment of a number of signaling molecules, leading to activation of pathways including kinases such as the mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) (Cowley et al., 1994; Xia et al., 1995). MAPK/ERK pathway modulates activities of many transcription factors, and thus regulates biological responses such as proliferation and differentiation. Accumulating data have shown that MAPK/ERK pathway plays a pivotal role in the neuroprotective effects of these neurotrophic factors (Takuma et al., 2000; Han and Holtzman, 2000; Zhu et al., 2002).

Huperzine A, a novel *Lycopodium* alkaloid isolated from the Chinese folk medicine *Huperzia serrata*, is a reversible and selective inhibitor of acetylcholinesterase (AChE) and has been used in clinical treatment of Alzheimer's disease in China (Xu et al., 1995; Wang et al., 2006). Our previous studies have shown that, besides inhibiting AChE, huperzine A possesses a broad range of neuroprotective activities (Wang and Tang, 2005). It has been reported that huperzine A can attenuate cognitive deficits and neuronal damage after transient global ischemia in gerbils (Zhou et al., 2001) and chronical cerebral hypoperfusion in rats (Wang et al., 2000). We recently found that huperzine A can increase NGF production in cultured astrocytes (Tang et al., 2005a) and protect SHSY5Y neuroblastoma cells against oxidative stress damage via promoting NGF production (Tang et al., 2005b). In this study, we attempt to investigate whether huperzine A exerts similar neurotrophic effects on transient cerebral ischemia and reperfusion-induced injury in mice and clarify the underlying mechanism.

2. Materials and methods

2.1. Materials

Huperzine A, provided by the Department of Phytochemistry in this Institute, is a colorless powder with m.p. 230 °C and purity >99%. It was dissolved and diluted in phosphate-buffered saline (PBS). NGF E_{max}® Immunoassay System, BDNF E_{max}® Immunoassay System, TGF-β_1 E_{max}® Immunoassay System and Reverse Transcription System were purchased from Promega (Madison, WI, USA). TRIzol reagent was purchased from Invitrogen (CA, USA). Rabbit anti-phospho-ERK 1/2 and rabbit anti-ERK 1/2 were purchased from Cell Signaling Technology (MA, USA). Mouse anti-GAPDH was purchased from KangChen (Shanghai, China). ECL plus Western blotting detection system was purchased from Amersham Biosciences (Piscataway, USA).

2.2. Animals

Male Kunming strain mice (SPF, certificate number SYXK [Shanghai] 2003-0029), weighing 18–22 g, were purchased from Shanghai Experimental Animal Centre, Chinese Academy of Science. Animals were maintained in colony cages under an ambient temperature of 22–25 °C, 50–60% relative humidity, with a 12-h light/dark cycle and free access to food and water. All procedures were carried out in compliance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China on November 14, 1988.

2.3. Surgery and drug administration

Mice were overnight fasted from food but allowed free access to water and then anesthetized with chloral hydrate (350 mg/kg, i.p.). Core body temperature was recorded using a rectal temperature probe and maintained at 37.0±0.5 °C using a heating lamp throughout the procedure. Via surgical incision, the right femoral artery was cannulated (PE 10, Becton Dickinson, Sparks, MD, USA) to allow measurement of mean arterial blood pressure (MAP). Operation was performed as described in previous reports (Zhao et al., 1998; Tang et al., 1998; Xu et al., 2004) with slight modifications. Bilateral common carotid arteries were exposed via a small ventral neck incision and occluded twice (10 min each) with microvascular clips and between the two periods of occlusion, there was a 15-min reperfusion (ischemia 10 min–reperfusion 15 min–ischemia 10 min). During the period of first occlusion, approximately distal 1 cm of tail was cut off and MAP was reduced to 42±2 mm Hg by withdrawing 0.3 ml of blood. After the operation, 1 ml saline was supplemented intraperitoneally. Sham-operated mice received the same surgical treatment without occlusions, tail cut, hemorrhage and saline supplement. Oral administration of huperzine A (0.2 mg/kg) or saline, once a day, was started 2 days before the surgery and terminated on the day of sacrifice. On the day of surgery, huperzine A was administrated 2 h prior to ischemia.

2.4. Water maze task

A plastic rectangular water maze (80 cm×50 cm×20 cm) used in previous report (Liu et al., 1998) was filled to height of 6 cm with water at 25±2 °C. The inner space of the water maze was separated by partitions to form an irregular path and four non-exits. The platform is invisible for the mouse until it swims to the end point of the path. Therefore, whether it can find the platform depends on learning and memory rather than visual

Table 1
Sense and antisense sequences of primers used in the RT-PCR reactions

Gene	5′ (sense) and 3′ (antisense) primer
β-Actin	5′-CCTGCGTCTGGACCTGGCTG-3′ (sense)
	5′-CTCAGGAGGAGCAATGATCT-3′ (antisense)
NGF	5′-CTTCAGCATTCCCTTGACAC-3′ (sense)
	5′-AGCCTTCCTGCTGAGCACACA-3′ (antisense)
BDNF	5′-AGGTGAGAAGAGTGATGACCA TCC-3′ (sense)
	5′-CAACATAAATCCACTATCTTCCCC-3′ (antisense)
TGF-β_1	5′-TGGACCGCAACAACGCCATCTATGAGAAAACC-3′ (sense)
	5′-TGGAGCTGAAGCAATAGTTGGTATCCAGGGCT-3′ (antisense)

cues. Mouse was placed on the water maze with nose towards the wall of the starting point and trained to find the platform through the right pathway. Each mouse received two training sessions daily for 7 consecutive days before surgery towards a criterion of finding the platform within 30 s and less than two errors of entering non-exit. When the training procedure ended, mice that came up to the criterion were chosen and randomly divided into three groups. At the seventh day post-surgery, behavioral test was performed 2 h after administration of huperzine A. Number of errors and time period of finding the platform were recorded. If a mouse failed to find the platform within 60 s, the time period was recorded as 60 s.

2.5. Morphology

Three or four mice chosen randomly from each group were anesthetized with chloral hydrate (350 mg/kg, i.p.) immediately after behavior test and then perfused transcardially with normal saline followed by 4% paraformaldehyde. Whole brains were separated and then post-fixed in the same paraformaldehyde at 4 °C, dehydrated and subsequently embedded in paraffin blocks. Coronal sections of 8 μM were stained with hematoxylin–eosin.

2.6. RT-PCR

Mice were sacrificed 3, 6 and 9 h after surgery, respectively. After the cerebral cortex and hippocampus were separated, total RNA was isolated using TRIzol reagent according to the manufacture's protocol and was quantified by absorbance at 260 nm. RNA purity was determined by the A260/A280 ratio (average >1.85). Total RNA of each sample was first reverse-transcribed into cDNA using Reverse Transcription System. PCR amplification was performed with reagents from Promega. The cDNA solution was amplified with primers (Table 1) based on the NGF, BDNF and TGF-β_1 sequences. Amplifications were performed as following: NGF: 30 cycles of 95 °C for 60 s, 60 °C for 45 s and 72 °C for 60 s; BDNF: 35 cycles of 95 °C for 30 s, 60 °C for 60 s and 72 °C for 120 s; TGF-β_1: 30 cycles of 95 °C for 30 s, 60 °C for 60 s and 72 °C for 120 s; β-actin: 25 cycles of 95 °C for 30 s, 57 °C for 45 s and 72 °C for 60 s. PCR products were normalized in relation to standards of β-actin and separated by 1.5% agarose gels containing 0.5 μg/ml ethidium bromide and photographed by an ultraviolet gel documentation system.

2.7. Extraction and measurement of NGF, BDNF and TGF-β_1

Mice were sacrificed immediately after behavioral test, and the brain cortex and hippocampus were quickly separated and kept in ice. Cortex and hippocampus were homogenized with an ultrasonic cell disrupter in 2 ml and 0.5 ml ice-cold lysis buffer [HEPES 25 mmol/l, $MgCl_2 \cdot 6H_2O$ 5 mmol/l, EDTA·2Na 5 mmol/l, pH 7.4, 0.5% (v/w) Triton X-100, DTT 5 mmol/l, PMSF 2 mmol/l, Pepstation A 10 μg/ml, Leupetion 10 μg/ml], respectively. The lysates were centrifuged at 10,000×g for 10 min at 4 °C and the supernatant solutions were collected. The supernatant from each sample was diluted five times with Dulbecco's PBS and acidified to pH 2.6. After 15 min of stay at room temperature, the diluted supernatants were neutralized to pH 7.6, of which the gross protein were measured by Coomassie blue protein binding method using bovine serum albumin as standard. NGF, BDNF and TGF-β_1 in the cerebral cortex and hippocampus were measured by a two-site enzyme-linked immunosorbent assay (ELISA) using NGF E_{max}®, BDNF E_{max}® and TGF-β_1 E_{max}® Immunoassay system, respectively, according to the manufacture's protocols.

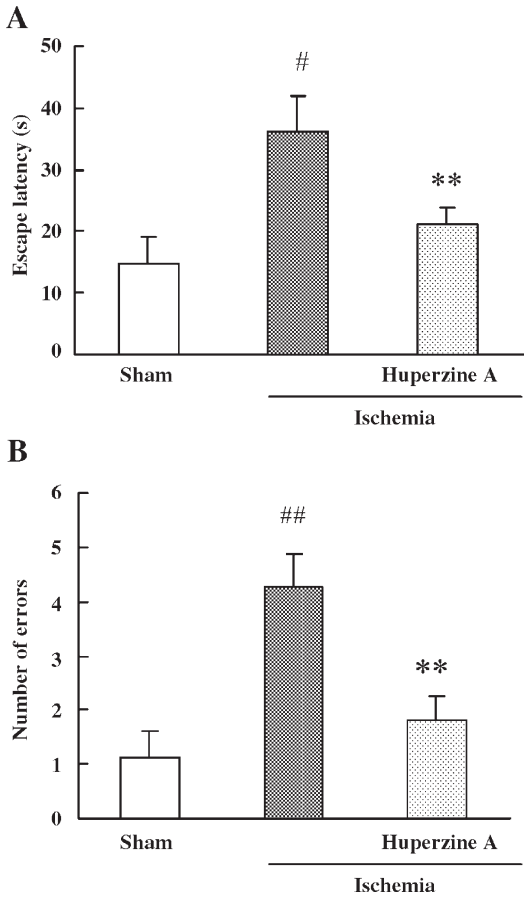

Fig. 1. Effects of huperzine A on the spatial performance deficits induced by transient cerebral ischemia and reperfusion in water maze. Mice were orally administrated with huperzine A (0.2 mg/kg/day, started 2 days before surgery and lasted for 7 days after surgery). Tests were performed at the seventh day after surgery. (A) Time to find the platform, (B) number of entering non-exit. Data were expressed as means±S.E.M., $n=12$, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. sham-operated group; $^{**}P<0.01$ vs. ischemia group.

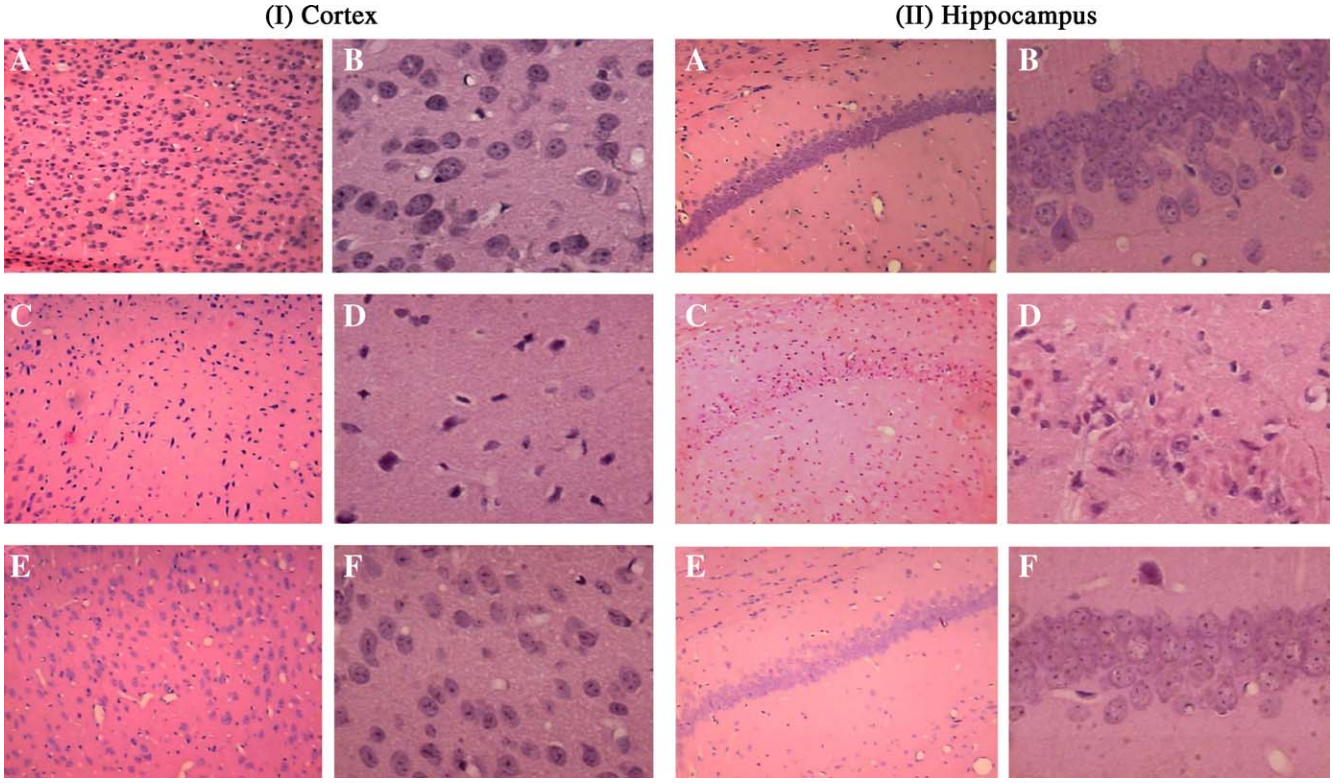

Fig. 2. Effects of huperzine A on morphologic changes induced by transient cerebral ischemia and reperfusion in mice (haematocylin and eosin staining). Mice were orally administrated with huperzine A (0.2 mg/kg/day, started 2 days before surgery and lasted for 7 days after surgery). (I) Frontal cortex; (II) CA1 region in hippocampus: (A, B) sham-operated mice; (C, D) mice subjected to transient cerebral ischemia and reperfusion; (E, F) mice subjected to transient cerebral ischemia and reperfusion plus treatment of huperzine A. Representative phase contrast micrographs: A, C, E: 100× magnification; B, D, F: 400× magnification.

2.8. Western blot analysis of phospho-ERK 1/2 and ERK 1/2

Mice were sacrificed 3 h, 24 h and 7 days after surgery, respectively. Cortex and hippocampus were homogenized with an ultrasonic cell disrupter in 1 ml and 0.3 ml ice-cold lysis buffer (as shown in the previous section), respectively. Lysates were centrifuged at 10,000×*g* for 10 min at 4 °C and the supernatant solutions were collected. The protein concentrations were determined by Coomassie blue protein binding method using bovine serum albumin as standard. Samples containing equal amounts of protein (50 μg) were boiled in protein loading buffer for 10 min, separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with TBST (Tris-buffered saline with 0.1% Tween) containing 5% non-fat milk, the membranes were kept at 4 °C overnight with primary antibodies to phospho-ERK 1/2, ERK 1/2 (1:1000 dilution) and GAPDH (1:8000 dilution), respectively, followed by HRP-conjugated anti-rabbit or anti-mouse IgG at room temperature for 2 h. The target protein bands were detected using the ECL plus Western blotting detection system and Kodak X-ray film.

2.9. Statistical analysis

Data were expressed as means ± 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. Effects of huperzine A on memory deficits induced by transient cerebral ischemia and reperfusion

Mice subjected to transient cerebral ischemia and reperfusion showed prolonged latency to find the platform (Fig. 1A) and increased errors of entering non-exit (Fig. 1B) ($P<0.01$, $P<0.05$ vs. sham-operated group) in the water maze task. These memory deficits were markedly attenuated by huperzine A at a dose of 0.2 mg/kg ($P<0.01$ vs. ischemia group).

3.2. Effects of huperzine A on morphologic alterations in the cerebral cortex and hippocampus induced by transient cerebral ischemia and reperfusion

As shown in Fig. 2, typical neuropathological changes were observed in the cerebral cortex and hippocampus at the seventh day after transient cerebral ischemia and reperfusion. Histological observation of the cerebral cortex and hippocampus in sham-operated mice showed that neurons were clear and moderate-sized with normal microstructure, while the corresponding brain regions in mice suffering from transient cerebral ischemia and reperfusion exhibited significant neuronal loss, shrinkage and dark staining. Neuronal death and loss were especially obvious in frontal cortex and CA1 region of hippocampus. These pathologic changes were prominently suppressed in mice treated with huperzine A.

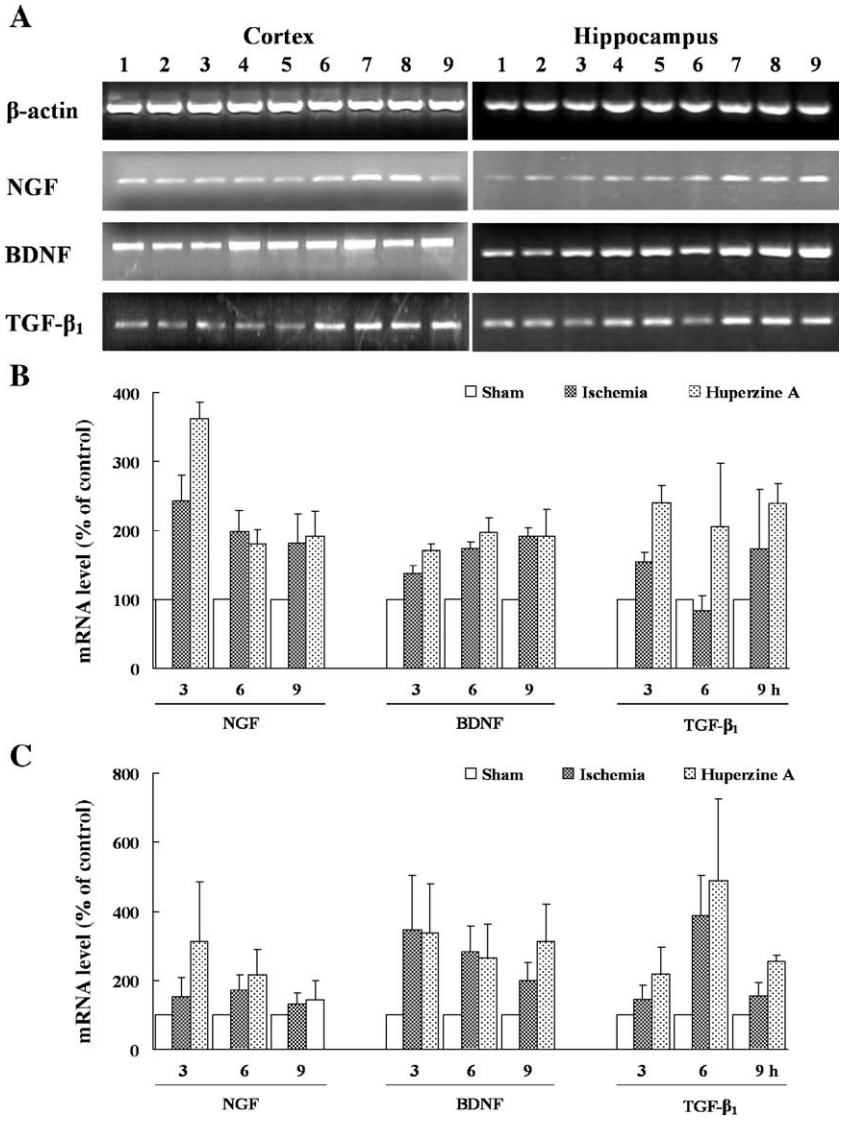

Fig. 3. Time course of NGF, BDNF and TGF-β_1 mRNA expressions in the cerebral cortex and hippocampus of mice. Total RNA was isolated and subjected to RT-PCR. (A) The PCR products were normalized by β-actin. Lanes 1–3: sham-operated mice in 3, 6, 9 h after reperfusion, respectively; lanes 4–6: ischemic mice in 3, 6, 9 h after reperfusion, respectively; lanes 7–9: ischemic mice treated with huperzine A (0.2 mg/kg/day) in 3, 6, 9 h after reperfusion, respectively. (B) Cortex and (C) hippocampus were quantitative summaries of results in (A). Data were means±S.E.M. expressed as percentage of control value, $n=4$.

3.3. Effects of huperzine A on the mRNA and protein levels of NGF, BDNF and TGF-β_1

For analysis of NGF, BDNF and TGF-β_1 mRNA levels, a comparative PCR approach was used to measure the target cDNAs amplified from the mRNA samples (Fig. 3). NGF, BDNF and TGF-β_1 mRNA levels were up-regulated at 3, 6 and 9 h after reperfusion in the cerebral cortex and hippocampus. Huperzine A at 0.2 mg/kg produced a more pronounced increase on the expression of NGF, BDNF and TGF-β_1 mRNA compared with transient cerebral ischemia and reperfusion group. However, these changes were not statistically significant.

The effects of huperzine A on products of these genes were confirmed by ELISA assay (Fig. 4). At the seventh day after surgery, the protein levels of NGF, BDNF and TGF-β_1 in the cerebral cortex and hippocampus of ischemic mice were almost similar to those of sham-operated group. However, they were enhanced by huperzine A (0.2 mg/kg) to measurably above the control level in the cerebral cortex and hippocampus ($P<0.01$, $P<0.05$ vs. sham-operated group).

3.4. Effects of huperzine A on phosphorylation of MAPK/ERK 1/2 kinases

The effects of huperzine A on phosphorylation of ERK 1/2 kinases in the cerebral cortex and hippocampus were determined by Western blot analysis (Fig. 5). Immunoblotting was performed with an antibody recognizing the ERK 1 and ERK 2 double phosphorylation on Thr 202 and Tyr 204 residues or with an antibody recognizing ERK 1/2 regardless of its phosphorylation state. Transient cerebral ischemia and reperfusion significantly increased ERK 1/2 phosphorylation in the cerebral cortex and hippocampus at 3 h, 24 h and 7 days after reperfusion ($P<0.01$, $P<0.05$ vs. sham-operated group). Increases of ERK

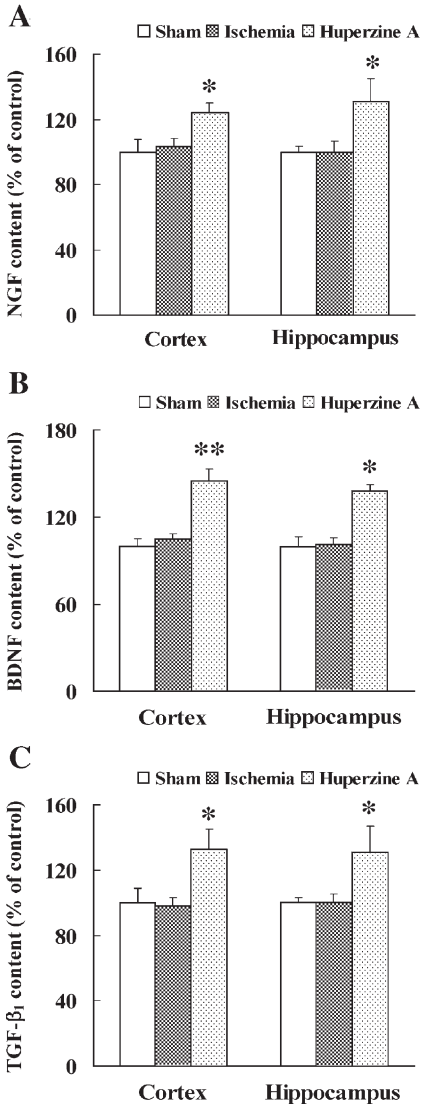

Fig. 4. Effects of huperzine A on NGF, BDNF and TGF-β_1 content in the cerebral cortex and hippocampus by ELISA assay. Mice were subjected to transient cerebral ischemia and reperfusion for 7 days. Subchronic oral administration of huperzine A (0.2 mg/kg/day) was started 2 days before surgery and lasted for 7 days after surgery. (A) The NGF content, (B) the BDNF content, (C) the TGF-β_1 content. Data were means±S.E.M. expressed as percentage of control value, $n=9$, *$P<0.05$, **$P<0.01$ vs. ischemia group.

1/2 phosphorylation in the cerebral cortex and hippocampus were more prominent in the huperzine A-treated group ($P<0.01$, $P<0.05$ vs. ischemia group). On the other hand, total ERK 1/2 proteins remained unaffected in the cerebral cortex and hippocampus throughout.

4. Discussion

Huperzine A has been found to exhibit a broad range of neuroprotective activities (Wang and Tang, 2005). In our recent studies focusing on the correlation between neurotrophic effects of huperzine A and NGF, we have observed that huperzine A can increase NGF secretion in astrocytes, induce neurotrophin-like activity in neuron-like PC12 cells (Tang et al., 2005a) and protect SHSY5Y neuroblastoma cells against oxidative stress damage via promoting NGF production (Tang et al., 2005b). These in vitro findings suggest that the neuroprotective effects of huperzine A are mediated at least partly by NGF production and its downstream signaling pathway. In the present study, using mice subjected to transient cerebral ischemia and reperfusion, we determined whether huperzine A possessed these neurotrophic effects in vivo and tried to clarify the potential downstream signaling pathway.

It is well known that delayed neuronal death follows transient cerebral ischemia in selective, vulnerable regions of the brain, especially in the hippocampus. The deficits in learning and memory induced by ischemia show a close correlation with neuronal death in the hippocampal CA1 region (Block, 1999). Consistent with the previous reports (Zhao et al., 1998; Tang et al., 1998; Xu et al., 2004), BCCAo combined with systemic hypotension induces ischemic insult in mice, resulting in marked amnesic effects along with signs of neurodegeneration, including memory deficits as shown by increased errors of entering non-exit and prolonged latency to find the platform and significant neuronal death and loss in the cerebral cortex and hippocampus especially in CA1 region. Our results indicate that neuronal damage might contribute to behavioral impairment in this ischemic model. Subchronic oral administration of huperzine A significantly attenuated transient cerebral ischemia and reperfusion-induced histological lesions in the brain and improved the water maze performance. These results support our previous studies (Zhou et al., 2001; Wang et al., 2000), and provide direct histopathological and behavioral evidence for the protective effects of huperzine A against transient cerebral ischemia and reperfusion. Such findings raise the important questions about the underlying mechanisms.

The neurotrophin family of growth factors, such as NGF, BDNF, NT_{3-5}, bind and activate specific tyrosine kinase (Trk) receptors to promote cell survival and growth of different cell populations. Therefore, growing attention has been paid to the use of neurotrophins as therapeutic agents against neurodegeneration. Numerous studies have documented that NGF and BDNF possess neuroprotective functions in the cerebral cortex and hippocampus, which are particularly vulnerable to cerebral ischemia (Shigeno et al., 1991; Pechan et al., 1995; Schabitz et al., 1997; Beck et al., 1994). Up-regulation of NGF and BDNF expression has been found in the brains of rats after middle cerebral artery occlusion (MCAO), and been considered to play an important role in the protection of ischemic injured neuronal cells (Kokaia et al., 1995). Neuroprotective effect of TGF-β_1 has been elucidated in many animal models of cerebral ischemia (Prehn et al., 1993; Gross et al., 1993; Henrich-Noack et al., 1996). This factor is minimally expressed in the intact brain and has been found to be strongly up-regulated in the central nervous system following ischemia-induced brain damage (Zhu et al., 2000). In light of these findings and the neurotrophic effects of huperzine A in vitro (Tang et al., 2005a,b), it is reasonable to propose that neurotrophic factors might participate in the neuroprotective effects of huperzine A against transient cerebral ischemia and reperfusion. The expression of neurotrophic factors in hippocampus and cerebral cortex has been shown to be

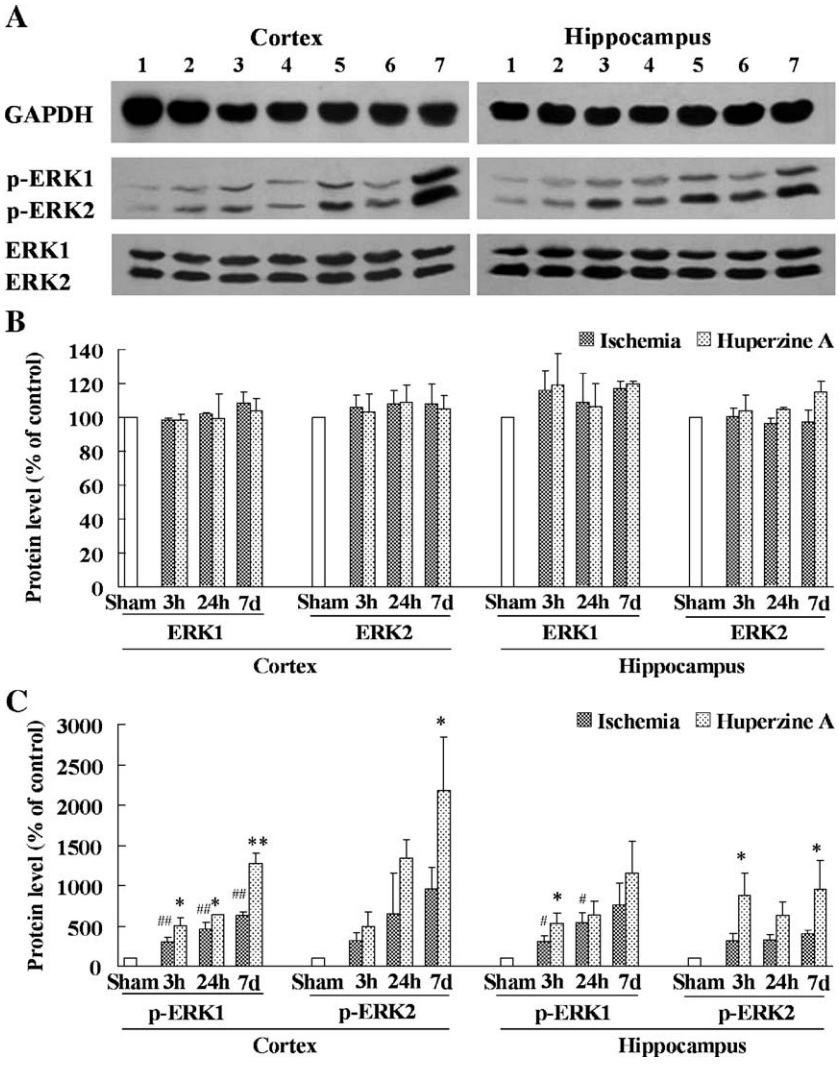

Fig. 5. Effects of huperzine A on ERK 1/2 and phospho-ERK 1/2 levels in the cerebral cortex and hippocampus by western blot. (A) The protein products were normalized by GAPDH. Lane 1: sham-operated mice; lanes 2, 4, 6: ischemic mice in 3 h, 24 h and 7 days after reperfusion, respectively; lanes 3, 5, 7: mice treated with huperzine A (0.2 mg/kg/day) in 3 h, 24 h and 7 days after reperfusion, respectively. (B) ERK 1/2 and (C) Phospho-ERK 1/2 were quantitative summaries of results in (A). Data were means ± S.E.M. expressed as percentage of control value, $n=5$, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. sham-operated group; $^{*}P<0.05$, $^{**}P<0.01$ vs. ischemia group.

up-regulated after ischemic insults and the elevated expression may play an important role in protection of ischemic injured neuronal cells (Tsukahara et al., 1998; Wei et al., 2000; Yanamoto et al., 2000). In accordance with these previous reports, we found that the NGF, BDNF and TGF-β_1 mRNA levels in the cerebral cortex and hippocampus were observed to immediately increase after reperfusion, but it was a transient and stress-activated response. Together with the findings that mice suffered from ischemia and reperfusion exhibited memory deficits and neuronal death, we think it was far from enough to survive from ischemic injury with this self-protection alone. Compared with stress-activated self-protection, however, huperzine A further and persistently increased neurotrophic factors production, and significantly attenuated behavioral and morphologic signs of damage. Since overexpressions of neurotrophic factors were thought to be protective against cerebral ischemic injury (Ding et al., 2004), we suggest that the capability of huperzine A to further increase NGF, BDNF and TGF-β_1 expressions may contribute to its neuroprotective effects against ischemic insult. Hence, the neurotrophic effects of huperzine A are verified in vivo.

NGF and BDNF bind to specific receptors, which are structurally related and belong to the Trk family of receptor tyrosine kinase, and these receptors mediate their biological responses to neurotrophins by activating multiple signaling pathways, including the activation of the MAPK isoforms ERK 1 and ERK 2 (Greene and Kaplan, 1995). MAPK/ERK pathway is also probably involved in the TGF-β_1 signaling (Massague, 2000). Dominant negative forms of Raf and MEK have been shown to block neurotrophin-induced neuritogenesis, whereas constitutively active forms of these elements promote neurite outgrowth in the absence of neurotrophins (Segal and Greenberg, 1996; Skaper and Walsh, 1998). These studies highlight the importance of MAPK/ERK pathway in neurotrophic signaling. More recent data have accumulated showing that phospho-ERK 1/2 is up-regulated in rodents after global ischemia and MCAO (Hu et al., 2000; Irving et al., 2000). The observation that phospho-ERK 1/2 levels increased in survival cells and decreased in dead cells after

focal and global ischemia suggested that activation of the ERK 1/2 "survival" pathway might be required to prevent cells succumbing to ischemia-induced death. Furthermore, abundant evidences indicate the important role of MAPK/ERK pathway in the neuroprotective effects of the neurotrophic factors against ischemia-induced injury (Han and Holtzman, 2000; Zhu et al., 2002). Consistent with previous reports (Hu et al., 2000; Irving et al., 2000), we found that the ERK 1/2 phosphorylation were significantly increased immediately after transient cerebral ischemia and reperfusion in both cerebral cortex and hippocampus, and lasted throughout the subsequent 7 days. This phenomenon might represent a stress reaction of self-protection. Oral administration of huperzine A could markedly enhance the phosphorylation of ERK 1/2, probably potentiating this protective effect. Our previous study showed that the neuroprotective effects of huperzine A against H_2O_2-induced oxidative stress were antagonized by the MAPK/ERK inhibitor, PD98059 (Tang et al., 2005b). With these in vitro and in vivo findings, it is reasonable to hypothesize that huperzine A increases NGF, BDNF and TGF-β_1 expressions, and enhances the intracellular signaling of these endogenously produced neurotrophic factors, resulting in protection against transient cerebral ischemia and reperfusion in mice.

In conclusion, the present study demonstrate that huperzine A can attenuate memory deficits and neuronal damage induced by transient cerebral ischemia and reperfusion in mice, and NGF, BDNF and TGF-β_1 are very likely to be related to the neuroprotective effects of huperzine A. Activation of the MAPK/ERK pathway might be important for the in vivo neurotrophic effects of huperzine A against transient cerebral ischemia and reperfusion.

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References

Beck T, Lindholm D, Castren E, Wree A. Brain-derived neurotrophic factor protects against ischemic cell damage in rat hippocampus. J Cereb Blood Flow Metab 1994;14:689–92.

Block F. Global ischemia and behavioural deficits. Prog Neurobiol 1999;58:279–95.

Choi DW, Rothman SM. The role of glutamate neurotoxicity in hypoxic–ischemic neuronal death. Annu Rev Neurosci 1990;13:171–82.

Cowley S, Paterson H, Kemp P, Marshall CJ. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 1994;77:841–52.

Ding Y, Li J, Luan X, Ding YH, Lai Q, Rafols JA, et al. Exercise preconditioning reduces brain damage in ischemic rats that may be associated with regional angiogenesis and cellular overexpression of neurotrophin. Neuroscience 2004;124(3):583–91.

Greene LA, Kaplan DR. Early events in neurotrophin signalling via Trk and p75 receptors. Curr Opin Neurobiol 1995;5:579–87.

Gross CE, Bednar MM, Howard DB, Sporn MB. Transforming growth factor-beta 1 reduces infarct size after experimental cerebral ischemia in a rabbit model. Stroke 1993;24:558–62.

Han BH, Holtzman DM. BDNF protects the neonatal brain from hypoxic–ischemic injury in vivo via the ERK pathway. J Neurosci 2000;20:5775–81.

Henrich-Noack P, Prehn JH, Krieglstein J. TGF-beta 1 protects hippocampal neurons against degeneration caused by transient global ischemia. Dose–response relationship and potential neuroprotective mechanisms. Stroke 1996;27:1609–15.

Hu BR, Liu CL, Park DJ. Alteration of MAP kinase pathways after transient forebrain ischemia. J Cereb Blood Flow Metab 2000;20:1089–95.

Irving EA, Barone FC, Reith AD, Hadingham SJ, Parsons AA. Differential activation of MAPK/ERK and p38/SAPK in neurons and glia following focal cerebral ischemia in the rat. Mol Brain Res 2000;77:65–75.

Kokaia Z, Zhao Q, Kokaia M, Elmer E, Metsis M, Smith ML, et al. Regulation of brain-derived neurotrophic factor gene expression after transient middle cerebral artery occlusion with and without brain damage. Exp Neurol 1995;136:73–88.

Liu J, Zhang HY, Tang XC, Wang B, He XC, Bai DL. Effects of synthetic (−)-huperzine A on cholinesterase activities and mouse water maze performance. Acta Pharmacol Sin 1998;19:413–6.

Massague J. How cells read TGF-beta signals. Nat Rev Mol Cell Biol 2000;1:169–78.

Pechan PA, Yoshida T, Panahian N, Moskowitz MA, Breakefield XO. Genetically modified fibroblasts producing NGF protect hippocampal neurons after ischemia in the rat. NeuroReport 1995;6:669–72.

Prehn JH, Backhauss C, Krieglstein J. Transforming growth factor-beta 1 prevents glutamate neurotoxicity in rat neocortical cultures and protects mouse neocortex from ischemic injury in vivo. J Cereb Blood Flow Metab 1993;13:521–5.

Schabitz WR, Schwab S, Spranger M, Hacke W. Intraventricular brain-derived neurotrophic factor reduces infarct size after focal cerebral ischemia in rats. J Cereb Blood Flow Metab 1997;17:500–6.

Schurr A, Rigor BM. The mechanism of cerebral hypoxic–ischemic damage. Hippocampus 1992;2:221–8.

Segal RA, Greenberg ME. Intracellular signaling pathways activated by neurotrophic factors. Annu Rev Neurosci 1996;19:463–89.

Shigeno T, Mima T, Takakura K, Graham DI, Kato G, Hashimoto Y, et al. Amelioration of delayed neuronal death in the hippocampus by nerve growth factor. J Neurosci 1991;11:2914–9.

Skaper SD, Walsh FS. Neurotrophic molecules: strategies for designing effective therapeutic molecules in neurodegeneration. Mol Cell Neurosci 1998;12:179–93.

Takuma K, Yoshida T, Lee E, Mori K, Kishi T, Baba A, et al. CV-2619 protects cultured astrocytes against reperfusion injury via nerve growth factor production. Eur J Pharmacol 2000;406:333–9.

Tang YP, Zhao SM, Sun CL, Jia XD, Hong QT, Hu JH. Effects of kangdai mixture on LPO, SOD and the cell in hippocampal CA1 region in cerebral ischemic mice treated by reperfusion. J Beijing Univ TCM 1998;21:23–6.

Tang LL, Wang R, Tang XC. Effects of huperzine A on secretion of nerve growth factor in cultured rat cortical astrocytes and neurite outgrowth in rat PC12 cells. Acta Pharmacol Sin 2005a;26:673–8.

Tang LL, Wang R, Tang XC. Huperzine A protects SHSY5Y neuroblastoma cells against oxidative stress damage via nerve growth factor production. Eur J Pharmacol 2005b;519:9-15.

Tsukahara T, Iihara K, Hashimoto N, Nishijima T, Taniguchi T. Increases in levels of brain-derived neurotrophic factor mRNA and its promoters after transient forebrain ischemia in the rat brain. Neurochem Int 1998;33:201–7.

Wang LM, Han YF, Tang XC. Huperzine A improves cognitive deficits caused by chronic cerebral hypoperfusion in rats. Eur J Pharmacol 2000;398:65–72.

Wang R, Tang XC. Neuroprotective effects of huperzine A: a natural cholinesterase inhibitor for the treatment of Alzheimer's disease. Neuro-Signals 2005;14:71–82.

Wang R, Yan H, Tang XC. Progress in studies of huperzine A, a natural cholinesterase inhibitor from Chinese folk medicine. Acta Pharmacol Sin 2006;27:1-26.

Wei G, Wu G, Cao X. Dynamic expression of glial cell line-derived neurotrophic factor after cerebral ischemia. Neuroreport 2000;11:1177–83.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326–31.

Xu SS, Gao ZX, Weng Z, Du ZM, Xu WA, Yang JS, et al. Efficacy of tablet huperzine A on memory, cognition, and behavior in Alzheimer's disease. Acta Pharmacol Sin 1995;16:391–5.

Xu RX, Wu Q, Shi JS. Experimental study of icariin on vascular dementia in rats induced by 2-vo method. Acta Pharmacol Sin 2004;25:1533–4.

Yagi T, Jikihara I, Fukumura M, Watabe K, Ohashi T, Eto Y, et al. Rescue of ischemia brain injury by adenoviral gene transfer of glia cell line-derived neurotrophic factor after transient global ischemia in gerbils. Brain Res 2000;885:273–82.

Yanamoto H, Mizuta I, Nagata I, Xue J, Zhang Z, Kikuchi H. Infarct tolerance accompanied enhanced BDNF-like immunoreactivity in neuronal nuclei. Brain Res 2000;877:331–44.

Zhao SM, Tang YP, Hong QT, Jia XD, Hu JH. Effects of kangdai mixture on cerebrovascular disturbance of learning and memorizing in mice. J Beijing Univ TCM 1998;21:41–2.

Zhou J, Zhang HY, Tang XC. Huperzine A attenuates cognitive deficits and hippocampal neuronal damage after transient global ischemia in gerbils. Neurosci Lett 2001;313:137–40.

Zhu Y, Roth-Eichhorn S, Braun N, Culmsee C, Rami A, Krieglstein J. The expression of transforming growth factor-beta 1 (TGF-beta 1) in hippocampal neurons: a temporary upregulated protein level after transient forebrain ischemia in the rat. Brain Res 2000;866:286–98.

Zhu Y, Yang GY, Ahlemeyer B, Pang L, Che XM, Culmsee C, et al. Transforming growth factor-beta 1 increases bad phosphorylation and protects neurons against damage. J Neurosci 2002;22:3898–909.