Neuroprotection against ischaemic brain injury by a GluR6-9c peptide containing the TAT protein transduction sequence

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It is well documented that N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors play a pivotal role in ischaemic brain injury. Recent studies have shown that kainate (KA) receptors are involved in neuronal cell death induced by seizure, which is mediated by the GluR6•PSD-95•MLK3 signalling module and subsequent c-Jun N-terminal kinase (JNK) activation. Here we investigate whether GluR6 mediated JNK activation is correlated with ischaemic brain injury. Our results show that cerebral ischaemia followed by reperfusion can enhance the assembly of the GluR6•PSD-95•MLK3 signalling module and JNK activation. As a result, activated JNK can not only phosphorylate the transcription factor c-Jun and up-regulate Fas L expression but can also phosphorylate 14-3-3 and promote Bax translocation to mitochondria, increase the release of cytochrome c and increase caspase-3 activation. These results indicate that GluR6 mediated JNK activation induced by ischaemia/reperfusion ultimately results in neuronal cell death via nuclear and non-nuclear pathways. Furthermore, the peptides we constructed, Tat-GluR6-9c, show a protective role against neuronal death induced by cerebral ischaemia/reperfusion through inhibiting the GluR6 mediated signal pathway. In summary, our results indicate that the KA receptor subunit GluR6 mediated JNK activation is involved in ischaemic brain injury and provides a new approach for stroke therapy.

Keywords: cerebral ischaemia; glutamate receptor 6 (GluR6); mixed lineage kinase-3 (MLK3); c-Jun N-terminal kinase (JNK); Tat protein

Abbreviations: AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; KA = kainate; IB = immunoblotting; IP = immunoprecipitation; NMDA = N-methyl-d-aspartate

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Introduction

The main mechanism underlying neuronal death in stroke is excitotoxicity, which is triggered by excessive activation of glutamate receptors [especially N-methyl-D-aspartate (NMDA) receptors] and subsequent calcium influx leading to increased intra-cellular calcium levels. Ionotropic glutamate receptors can be divided into NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate (KA) (Seeburg, 1993; Hollmann and Heinemann, 1994). Although the bulk of the evidence suggests that NMDA receptors may play a pivotal role in ischaemia-induced neuronal death, the classical hypothesis on NMDA receptor-mediated excitotoxicity faces the following challenges: first, the wide distribution of NMDA receptors can hardly account for the selective neuronal death after transient cerebral ischaemia (Gill and Lodge, 1997; Weiss and Sensi, 2000). Second, NMDA receptor antagonists confer robust
neuroprotection in animal models of focal (but not global) cerebral ischaemia; however, severe side-effects of the NMDA receptor blocker were demonstrated, and therefore, limited its application in clinical management of neurodegenerative diseases (Olney et al., 1989; Gill and Lodge, 1997). Third, in contrast to NMDA receptor antagonists, non-NMDA receptor antagonists exhibit a more protective role against neuron death in hippocampus CA1 subfields induced by transient global ischaemia (Chung et al., 2000). These findings raise new questions regarding the function of NMDA receptors in cerebral ischaemia/reperfusion and reinforce concerns about the potential roles of other glutamate receptors in mediating neuronal injury during cerebral ischaemia. In contrast to NMDA and AMPA receptors, little is known about the function of KA receptors in response to ischaemia. A recent study has shown that c-Jun N-terminal kinase (JNK)-deficient mice have strong resistance to the KA-induced seizure and hippocampal neuron apoptosis (Yang et al., 1997). Together with Mulle’s study (Mulle et al., 1998) showing that GluR6-deficient mice exhibit resistance to neurotoxic effects induced by KA, in turn, there is raised the possibility that the KA receptor subunit GluR6 may mediate the activation of JNK3 in response to excitotoxicity of glutamate. Studies indicated that the motif RLPGKETMA of the carboxylic terminal of GluR6 could bind to the PDZ1 domain of postsynaptic density protein 95 (PSD-95/SAP90) through a specific interaction (Garcia et al., 1998; Mehta et al., 2001). Thus, there may exist the triplicate complex GluR6/C15/PSD-95/C15/MLK3, which could form a signalling module to facilitate JNK activation. Our previous studies demonstrated that JNKS can be activated during cerebral ischaemia/reperfusion (Gu et al., 2001; Tian et al., 2003a); however, NMDA and AMPA receptors did not mediate JNK activation (Tian et al., 2003a). Thus, it renders us to think of the possible contribution of KA receptors to the activation of JNK in cerebral ischaemia/reperfusion.

In the present study, we show that Tat-GluR6-9c, a GluR6 C-terminus containing peptide conjugated to Tat peptide, could perturb the interaction of GluR6 with PSD-95 and suppress the assembly of the GluR6/C15/PSD-95/C15/MLK3 signalling module and, therefore, result in blockage of brain injury caused by a variety of noxious stimuli including cerebral ischaemia. (Fig. 1A)

Material and methods
Ischaemic model
Adult male Sprague–Dawley rats weighing 200–250 g were used. The experimental procedures were approved by the local legislation for ethics of experiments on animals. Transient cerebral ischaemia was induced by four-vessel occlusion (4-VO) as described before (Pulsinelli and Brierley, 1979). Briefly, rats were anaesthetized with chloral hydrate (300 mg/kg, i.p.) and both vertebral arteries were occluded permanently by electrocautery. Rats were allowed to recover for 24 h and fasted overnight. On the following day, both carotid arteries were occluded with aneurysm clips to induce cerebral ischaemia. After 15 min of the occlusion, the aneurysm clips were removed for reperfusion. Rats that lost their righting reflex and whose pupils were dilated and unresponsive to light were selected for the experiments. Rectal temperature was maintained at 36.5–37.5°C throughout the procedure. Rats with seizures were discarded. An EEG was monitored to ensure isoelectricity after carotid artery occlusion. Sham control was performed using the same surgical procedures except that the carotid arteries were not occluded.

Administration of peptides
Peptides (100 μg) or control peptides in 10 μl saline were administered to the rats 40 min before or 1 h after ischaemia through cerebral ventricular injection (antero posterior, 0.8 mm; lateral, 1.5 mm; depth, 3.5 mm from bregma).

Sample preparation
Rats were decapitated immediately after different times of reperfusion and then the hippocampi CA1 were isolated and quickly frozen in liquid nitrogen. The hippocampi were homogenized in an ice-cold homogenization buffer containing 50 mM 3-(N-morpholino)propanesulphonic acid (MOPS) (Sigma; pH 7.4), 100 mM KCl, 320 mM sucrose, 50 mM NaF, 0.5 mM MgCl₂, 0.2 mM DTT, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄ (Sigma), 20 mM sodium pyrophosphate, 20 mM β-phosphoglycerol, 1 mM p-nitrophenyl phosphate (PNPP), 1 mM benzamidine, 1 mM phenylmethylsulphonylfluoride (PMSF) and 5 μg/ml each of leupeptin, aprotinin and pepstatin A. The homogenates were centrifuged at 800 g for 10 min at 4°C. Supernatants were collected and centrifuged at 100 000 g for 30 min at 4°C. The supernatants were carefully removed and 500 μl homogenization buffer containing 1% Triton X-100 was added to the pellets, which then exposed to ultrasound. Protein concentration was determined by the methods of Lowry et al. Samples were stored at −80°C until use.

When necessary, the hippocampal CA1 was immediately isolated to prepare mitochondrial fractions. All procedures were conducted in a cold room. Non-frozen brain tissue was used to prepare mitochondrial fractions because freezing tissue causes release of cytochrome c from mitochondria. The hippocampal CA1 tissues were homogenized in 1 : 10 (w/v) ice-cold homogenization buffer. The homogenates were centrifuged at 800 g for 10 min at 4°C. The pellets were discarded, and supernatants were centrifuged at 17 000 g for 20 min at 4°C to get the cytosolic fraction in the supernatants and the crude mitochondrial fraction in the pellets. The protein concentrations were determined by the method of Lowry et al.

Nuclei extraction
The homogenates were centrifuged at 800 g for 10 min at 4°C. Supernatants as the cytosol part were collected and protein concentrations were determined. The nuclear pellets were extracted with 20 mM HEPES, pH 7.9, 20% glycerol, 420 mM NaCl, 0.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and enzyme inhibitors for 30 min at 4°C with constant agitation. After centrifugation at 12 000 g for 15 min at 4°C, supernatants as nuclear parts were collected and protein concentrations were determined. Samples were stored at −80°C and were thawed only once.
Immunoprecipitation

Tissue homogenates (400 μg of protein) were diluted 4-fold with 50 mM HEPES buffer, (pH 7.4), containing 10% glycerol, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40 and 1 mM each of EDTA, EGTA, PMSF and Na3VO4. Samples were preincubated for 1 h with 20 μl protein A Sepharose CL-4B (Amersham, Uppsala, Sweden) at 4°C, and then centrifuged to remove proteins adhered non-specifically to protein A. The supernatants were incubated with 1–2 μg primary antibodies for 4 h or overnight at 4°C. Protein A was added to the tube for another 2 h incubation. Samples were centrifuged at 10 000 g for 2 min at 4°C and the pellets were washed with immunoprecipitation (IP) buffer for three times. Bound proteins were eluted by boiling at 100°C for 5 min in SDS-PAGE loading...
buffer and then isolated by centrifuge. The supernatants were used for immunoblot analysis.

**Immunoblot**

Equal amounts of protein (100 μg/lane) were separated on polyacrylamide gels and then electrotransferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK). After blocking for 3 h in Tris-buffered saline with 0.1% Tween-20 (TBST) and 3% bovine serum albumin (BSA), membranes were incubated overnight at 4°C with primary antibodies in TBST containing 3% BSA. Membranes were then washed and incubated with alkaline phosphatase conjugated secondary antibodies in TBST for 2 h and developed using NBT/BCIP colour substrate (Promega, Madison, WI, USA). The densities of the bands on the membrane were scanned and analysed with an image analyser (LabWorks Software, UVP Upland, CA, USA).

**Hippocampal cell culture**

Neurons from hippocampi of fetal Sprague-Dawley rat (18 days gestation) were prepared as described previously with a little modification. Briefly, hippocampi were meticulously isolated in ice-cold high-glucose Dulbecco’s modified Eagle medium (h-DMEM, GibcorBRL, Grand Island, NY, USA). Hippocampal cells were dissociated by trypsinization [0.25% (w/v) trypsin and 0.05% EDTA in Ca2+- and Mg2+-free Hanks balanced salt solution] at 37°C for 15 min, followed by gentle triturating in plating medium (h-DMEM supplemented with 10% fetal bovine serum and 10% horse serum, Gibco BRL). Cells were seeded onto poly-l-lysine-coated wells (Sigma, St. Louis, MO, USA) or coverslips at a density of 0.8 × 10^5 cells per cm² and incubated at 37°C in 5% CO2 atmosphere. After 18–24 h, cells were incubated in Neurobasal Medium supplemented with B-27 (Gibco BRL) and 0.5 mM glutamine, and then half-replaced twice every week. Cultures were used after 16 days in vitro.

**Electrophysiological recordings**

The electrophysiological recordings were performed in the conventional whole-cell patch-recording configuration under voltage-clamp conditions. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PP-830, Narishige, Tokyo, Japan). The resistance between the recording electrode filled with pipette solution and the reference electrode was 4–6 MΩ. The standard external solution contained (mM): NaCl 150, KCl 5, MgCl2 1, CaCl2 2, HEPES 10 and glucose 10. The pH was adjusted to 7.4 with Tris-base. The osmolarity was adjusted to 310–320 mOsm/l with sucrose. The ionic composition of the internal solution medium was (mM): CsCl 140, CaCl2 0.5, MgCl2 2, EGTA 5, Na2ATP 4 and HEPES 10 with pH adjusted to 7.2, osmolarity adjusted to 280–300 mOsm/l. Membrane currents were measured using a patch-clamp amplifier (Axon 200B, Axon Instruments, Foster City, CA, USA), sampled and analysed using a Digidata 1322A interface and a personal computer with Clampex and Clampfit software (Version 9.0.1, Axon Instruments). In most experiments, 60–70% series resistance was compensated. The membrane potential was held at –60 mV throughout the experiment. All the experiments were carried out at room temperature (22–25°C).

**Histology and immunohistochemistry**

Rats were perfusion-fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) under anaesthesia after 5 days of ischaemia/reperfusion. Brains were removed quickly and further fixed with the same fixation solution at 4°C overnight. Post-fixed brains were embedded by paraffin, followed by preparation of coronal sections 5 μm thick using a microtome. The paraffin embedded brain sections were deparaffinized with xylene and rehydrated by ethanol at graded concentrations of 100–70% (v/v), followed by washing with water. The sections were stained with 0.1% (w/v) cresyl violet and were examined with light microscopy and the number of surviving hippocampal CA1 pyramidal cells per 1 mm length was counted as the neuronal density.

Immunoreactivity was determined by the avidin–biotin–peroxidase method. Briefly, sections were deparaffinized with xylene and rehydrated by ethanol at graded concentrations and distilled water. High-temperature antigen retrieval was performed in 1 mM citrate buffer. To block endogenous peroxidase activity, sections were incubated for 30 min in 1% H2O2. After being blocked with 5% (v/v) normal goat serum in PBS for 1 h at 37°C, sections were incubated with rabbit polyclonal antibodies against Fas L (1:100) or mouse monoclonal antibody against p-c-Jun (1:50) at 4°C 2 day. These sections were then incubated with biotinylated goat-anti-rabbit/mouse secondary antibody overnight and subsequently with avidin–conjugated horseradish peroxidase for 1 h at 37°C. Finally, sections were incubated with peroxidase substrate diaminobenzidine (DAB) until desired stain intensity developed.

**TUNEL staining**

TUNEL staining was performed using an ApopTag® Peroxidase In Situ Apoptosis Detection Kit according to the manufacturer’s protocol with minor modifications. The paraffin-embedded coronal sections were deparaffinized and rehydrated, and then treated with protease K at 20 μg/ml for 15 min at room temperature. Sections were incubated with reaction buffer containing TdT enzyme and at 37°C for 1 h. After washing with stop/wash buffer, sections were treated with anti-digoxigenin conjugate for 30 min at room temperature and subsequently developed colour in peroxidase substrate. The nuclei were lightly counterstained with 0.5% methyl green.

**Antibody and reagents**

The following primary antibodies were used: goat polyclonal anti-Grp78 (sc-7618), mouse monoclonal anti-p-JNKs (sc-6254), rabbit polyclonal anti-MLK3 (sc-13072), mouse monoclonal anti-p-c-Jun (sc-822), rabbit polyclonal anti-Fas L (sc-6237), rabbit polyclonal anti-Fas (sc-716), rabbit polyclonal anti-c-Jun (sc-1694), rabbit polyclonal anti-14-3-3 (sc-1019), rabbit polyclonal anti-NR2B (sc-9057) and rabbit polyclonal anti-actin (sc-10731) were purchased from Santa Cruz Biotechnology. Monoclonal antibody to phosphoserine was obtained from Alexis Biochemicals. Rabbit polyclonal anti-Bax, rabbit polyclonal anti-cytchrome c, rabbit polyclonal anti-caspase-3 and rabbit polyclonal anti-p-MLK3 were obtained from Cell Signal Biotechnology. Mouse monoclonal anti-PSD-95 (CPS35-100UL) was bought from Oncogene. Monoclonal antibody of cytochrome c oxidase subunit IV was obtained from Molecular Probes. Rabbit polyclonal anti-JNK3 antibody (06-749) was obtained from Upstate Biotechnology. The secondary antibodies used in our experiment were goat anti-mouse IgG, goat anti-rabbit IgG and donkey anti-goat IgG. They were from Sigma. ApopTag® Peroxidase In Situ Apoptosis Detection Kit (S7100) was purchased from Chemicon.
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Statistics
Values were expressed as mean ± SD and obtained from six independent rats. Statistical analysis of the results was carried out by Student’s t-test or one-way analysis of the variance (ANOVA) followed by the Duncan’s new multiple range method or Newman-Keuls test. P-values <0.05 were considered significant.

Results
Effects of the peptides in vitro and in cultured hippocampal neurons
In order to interfere with the interaction of GluR6 with PSD-95, we constructed a peptide comprising the nine COOH-terminal residues of GluR6 (Arg-Leu-Pro-Gly-Lys-Glu-Thr-Met-Ala; GluR6-9c), which was conserved in humans and rodents. Tat protein (Tyr-Gly-Arg-Lys-Arg-Gln-Arg-Arg-Arg), which was obtained originally from the cell-membrane transduction domain of the human immunodeficiency virus-type 1 (HIV-1), was fused to GluR6-9c and resulted in a 20-amino acid fusion peptide (Tat-GluR6-9c). We further examined whether Tat-GluR6-9c could be delivered into hippocampal neurons. The fluorophore dansyl chloride was conjugated to Tat-GluR6-9c and to HIV-1 Tat residues 38 to 48 (Lys-Ala-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-Lys-Lys; Tat38-48) outside of the transduction domain as a control peptide, respectively. These peptides were incubated with cultured hippocampal neurons and their fluorescence was visualized by fluoroscope. Neurons treated with Tat-GluR6-9c showed only background signal indicating no peptide uptake (Fig. 1B, right). Tat-GluR6-9c, Tat38-48, the Tat transduction sequence conjugated to two alanine residues (Tat-AA), or a Tat-GluR6-9c peptide in which the COOH-terminal ETMA motif contained four alanine residues (Tat-AA), or a Tat-GluR6-9c peptide in which the ETMA motif were used to examine the effect of the peptides on KA-induced whole-cell currents in cultured hippocampal neurons (Fig. 2A). Meanwhile, the intracellular Tat-GluR6-9c peptide also showed no effect on KA receptors-mediated currents during or after 15 min incubation of the peptides (Fig. 2B). In order to further confirm the results, we examine the KA-induced whole-cell currents with the peptides applied from the internal pipette solution. The results also show that the currents were not affected by the peptides (Fig. 2C). The above data indicate that Tat-GluR6-9c peptide will not affect KA receptors-mediated currents in spite of the fact that it can interrupt the interactions of GluR6 and PSD-95.

The peptides suppress the assembly of the GluR6•PSD-95•MLK3 signalling module and inhibit the activation of MLK3 and JNK3
We make the hypothesis that the assembly of the GluR6•PSD-95•MLK3 signalling module during cerebral ischaemia-reperfusion can facilitate the activation of JNK signalling pathway. A GluR6 c-terminus containing peptide could perturb the interaction of GluR6 with PSD-95 and suppress the assembly of the GluR6•PSD-95•MLK3 signalling module and subsequently prevent the activation of JNK as well as neuronal death induced by cerebral ischaemia-reperfusion.

To test our hypothesis, the peptides were administrated to the adult Sprague–Dawley rats through cerebral ventricular injection 40 min before ischaemia. Because JNK was selectively activated in the CA1 subfields of the hippocampus, the CA1 regions were isolated for further examinations (Gu et al., 2001). After various time of reperfusion, the interactions of GluR6 and MLK3 with PSD-95 and the phosphorylation of MLK3, JNK3 and c-Jun were examined, respectively. In our previous study, we found that the interaction of GluR6 and MLK3 with PSD-95 reached its peak level at 6 h reperfusion after 15 min ischaemia (Tian et al., 2005). In order to investigate whether pretreatment of rats with these peptides could affect the interaction of GluR6 and MLK3 with PSD-95, IP and immunoblotting (IB) were used to examine the association of GluR6 and MLK3 with PSD-95 after 15 min ischaemia followed by 6 h reperfusion, reciprocal IP experiments were carried out to confirm the results. As shown in Fig. 3A, the interaction of GluR6 and MLK3 with PSD-95 increased after 15 min ischaemia followed by 6 h reperfusion. Administration of Tat-GluR6-9c 40 min prior to ischaemia diminished the increased interaction of GluR6, MLK3 with PSD-95, meanwhile the protein level of GluR6, PSD-95 and MLK3 were not altered. Conversely, the same dose of control peptides Tat-GluR6AA did not affect the increased associations of GluR6, MLK3 with PSD-95.
Previous studies indicated that MLK3, an upstream kinase of JNK, could be activated via GluR6 and PSD-95 (Savinainen et al., 2001). We then analysed the effects of the peptides on the activation of MLK3. IB was used to examine the phosphorylation of MLK3. As shown in Fig. 3B, 6 h reperfusion after 15 min ischaemia resulted in remarkable increase in the phosphorylation of MLK3. Pretreatment of Tat-GluR6-9c significantly diminished the increase in the phosphorylation of MLK3, while the protein level of MLK3 was not changed. The same dose of control peptides Tat-GluR6AA did not affect the increased phosphorylation of MLK3. We have reported that JNK activation was biphasically increased during transient cerebral ischaemia followed by reperfusion (Gu et al., 2001). To further determine whether the activation of JNK pathway downstream of MLK3 was affected by the peptides after cerebral ischaemia followed by reperfusion, we examined the phosphorylation of JNK at two peak levels: 30 min and 3 days after ischaemia. Results of western blotting revealed that activation of JNK3 after 3 days, but not 30 min of reperfusion was suppressed by application of the peptides Tat-GluR6-9c (Fig. 3C). Furthermore, similar results were obtained from c-Jun, one of the substrates of JNK, as compared with that of MLK3 and JNK3 (Fig. 3D). As mentioned above, our results suggest that MLK3 and JNK3 activation induced by ischaemia/reperfusion were mediated by GluR6/PSD-95/MLK3 signalling module. Tat-GluR6-9c could perturb the interaction of GluR6 with PSD-95 and, therefore, inhibit the assembly of the GluR6/PSD-95/MLK3 signalling module and, as a result, prevent the JNK3 activation and translocation into the nucleus and subsequently suppress the phosphorylation of c-Jun. At the same time, results from immunohistochemistry also confirmed that pretreatment with Tat-GluR6-9c could significantly diminish the immunoreactivity of p-c-Jun in the nucleus of hippocampal CA1 compared with 6 h reperfusion groups and the control peptide Tat-GluR6-AA-treated groups (Fig. 3E).

**Inhibition of JNK activity by the peptides diminished the increased expression of Fas L induced by cerebral ischaemia-reperfusion in the hippocampal CA1 region**

To investigate whether the Fas receptor-mediated pathway is involved in the apoptotic profile during cerebral ischaemia/reperfusion, the expression of Fas L and Fas was analysed by western blot. As indicated in Fig. 4A, the expression of Fas L was significantly increased 6 h after reperfusion. However, application of Tat-GluR6-9c could diminish the increasing expression of Fas L induced by cerebral ischaemia and reperfusion. The same dose of control peptide Tat-GluR6-9c did not affect the increase on the expression of Fas L. The protein level of Fas was not affected by Tat-GluR6-9c and control peptide. As shown in Fig. 4B, the results of immunohistochemistry also revealed that weak Fas L immunoreactivity was detected in the cytosol of hippocampal CA1 in the sham group (a, e). On the contrary, Fas L immunoreactivity was significantly increased after 6 h reperfusion (b, f) compared with the sham group. No inhibitory effects of control peptide Tat-GluR6AA on Fas L immunoreactivity were detected (c, g). However, Fas L immunoreactivity after ischaemia 6 h reperfusion was significantly inhibited by application of Tat-GluR6-9c (d, h).

**Tat-GluR6-9c attenuated the decreased interaction of Bax and 14-3-3, phosphorylation of 14-3-3, and neuronal apoptosis in hippocampal CA1 induced by cerebral ischaemia-reperfusion**

To elucidate the involvement of mitochondria-mediated pathway in the apoptotic programme during global ischaemia/reperfusion, phosphorylation of 14-3-3, the interaction of Bax and 14-3-3, and the expression of Bax and cytochrome c in mitochondria and cytosol after brain ischaemia was examined by IB and IP. As indicated in Fig. 5A, results of reciprocal IP showed that the phosphorylation of 14-3-3 was significantly increased at 6 h reperfusion after 15 min of ischaemia, but the protein level of 14-3-3 was not affected at various times after 15 min of ischaemia. Meanwhile, the association of Bax and 14-3-3 keeps decreasing in company with the phosphorylation of 14-3-3. The disassociated Bax would translocate from cytosol to mitochondria and facilitate cytochrome c release which ultimately causes caspase-3 activation and results in apoptosis.

Recent studies indicated that JNK could phosphorylate 14-3-3 protein and promote Bax disassociate from 14-3-3.
and translocate to mitochondria. Since Tat-GluR6-9c could inhibit the activation of JNK, we wondered whether the inhibition of JNK signalling pathway could attenuate the decreased interaction of Bax and 14-3-3, phosphorylation of 14-3-3 and subsequently prevent Bax translocation, the release of cytochrome c and caspase3 activation. As shown in Fig. 5B, the inhibitory effects of Tat-GluR6-9c on the phosphorylation of 14-3-3 and the association of Bax and 14-3-3 can be observed at 6 hours reperfusion compared with the control peptide Tat-GluR6-AA-treated groups. Similar inhibitory effects on the Bax translocation can be obtained, the increased Bax content in the mitochondrial fraction was attenuated by the pretreatment with Tat-GluR6-9c (Fig. 5C).

In the mitochondrial fraction cytochrome c immuno-reactivity was evident as a single band of molecular mass of 15 kDa. However, it was barely detected in the sham CA1 subregion (Fig. 5D). A significant amount of mitochondrial cytochrome c was detected in the controls and it decreased

**Fig. 3** Continued
at 6 h reperfusion after ischaemia, corresponding to a marked increase in the cytosolic fraction at 6 h reperfusion (Fig. 5D). Moreover, Tat-GluR6-9c also can inhibit the release of cytochrome c to cytosol compared with 6 h reperfusion groups and the control peptide Tat-GluR6-AA-treated groups (Fig. 5D). To further validate whether other mitochondrial protein was released from mitochondria, we examined the cytochrome c oxidase level in the cytosolic and mitochondrial fraction using anti-cytochrome c oxidase subunit IV antibody. The cytochrome c oxidase subunit IV was detected only in the mitochondrial fraction, not in the cytosolic fraction in sham, ischaemia and application of peptides, which suggests that cytochrome c oxidase was not related with the release of cytochrome from mitochondria.

In line with the demonstrated inhibitory effects on the mitochondrial signal pathway, Tat-GluR6-9c treatment also can diminish the activation of caspase-3. The activation of caspase-3 was confirmed at 6 h reperfusion after ischaemia by the methods of immunoblot with antibodies recognizing the activated fragments for caspase-3 (Fig. 5E). Administration of Tat-GluR6-9c can significantly suppress the activation of caspase-3 induced by ischaemia/reperfusion.

Since caspase-3 plays a critical role in ischaemic neuronal apoptosis in the hippocampal CA1 subfield, TUNEL staining was performed to further explore the role of Tat-GluR6-9c in ischaemia-induced apoptosis. Rats were pretreated with Tat-GluR6-9c or Tat-GluR6-AA by cerebral ventricular injection 40 min before ischaemia. After 3 days reperfusion, rats were perfusion-fixed with paraformaldehyde and TUNEL staining was used to examine the apoptosis of CA1 pyramidal cells in hippocampus. As shown in Fig. 5F, a significant number of TUNEL-positive cells were observed 3 days after ischaemia (b, f), and rats subjected to 15 min of ischaemia followed by 6 h of reperfusion with administration of 100 μg of Tat-GluR6AA (c, g), 100 μg of Tat-GluR6-9c 40 min before ischaemia (d, h). Data were obtained from six independent animals in each experimental group, and the results of a typical experiment are presented. Boxed areas in left column are shown at higher magnification in right column. a, b, c, d: ×40; e, f, g, h: ×400. Scale bar in D = 200 μm; bar in H =10 μm.

### Neuroprotective role of the peptides on cerebral ischaemia in vivo and KA stimulation in vitro

To investigate whether pretreatment of Tat-GluR6-9c would have neuroprotection on ischaemia-induced cell death, adult S-D rats were subjected to 15 min ischaemia followed by 5 days...
reperfusion. Rats were pretreated with Tat-GluR6-9c or Tat-GluR6AA by cerebral ventricular injection 40 min before ischaemia. After 5 days reperfusion, rats were perfusion-fixed with paraformaldehyde and cresyl violet staining was used to examine the survival of CA1 pyramidal cells in hippocampus. Results from histology indicated that normal CA1 pyramidal cells showed round and pale stained nuclei (Fig. 6A and E), while ischaemia-induced dead cells showed pyknotic nuclei (Fig. 6B and F). Administration of Tat-GluR6-9c 40 min before cerebral ischaemia significantly decreased neuronal degeneration (Fig. 6D and H). At the same time, as the control, Tat-GluR6AA did not show any protection against the degeneration induced by ischaemia and 5 days reperfusion (Fig. 6C and G). The neuronal density of sham group, ischaemia insulted group, Tat-GluR6AA and Tat-GluR6-9c treated group were 205.0 ± 27.3, 28.2 ± 6.4, 33.7 ± 5.1 and 93.4 ± 14.6, respectively.

Since Tat-GluR6-9c could perturb the interaction of GluR6 with PSD-95 and in turn protect neurons against the ischaemic damage, we wonder if it could act as a protective trouper in KA induced neuronal degeneration. Further study was explored to investigate the role of Tat-GluR6-9c in KA induced apoptotic neuronal death. As shown in Fig. 7, 100 μM KA in cultured hippocampal neurons induced severe apoptotic cell death compared with sham control. However, administration of Tat-GluR6-9c (50 nM) 1 h before kainic acid stimulation could attenuate the apoptotic cell death determined by DAPI staining. On the contrary, Tat-GluR6AA could not prevent cultured hippocampal neurons from apoptosis induced by KA. In order to exclude the possible role of NMDA receptor, the cultured hippocampal neurons were pretreated with MK-801, an antagonist of NMDA receptor. The results show that MK-801 failed to rescue neurons from apoptosis induced by KA.

Discussion

Here we report for the first time that the KA receptor subunit GluR6 plays an important role in ischaemia-induced JNK3 activation and neuronal cell death. Although studies by Savinainen and colleagues indicated that GluR6, PSD-95 and MLK3 form a signalling module and facilitate MLK3 and JNK phosphorylation and activation (Savinainen et al., 2001), it is still unclear whether inhibition of the assembly of GluR6•PSD-95•MLK3 signalling module could attenuate
JNK activation and brain damage induced by ischaemia/reperfusion. Our previous studies demonstrated that cerebral ischaemia/reperfusion facilitates the assembly of the GluR6-PSD-95-MLK3 signalling module and the activation of MLK3 and JNK3. In the present study, we construct a peptide comprising the nine C-terminal amino acids of GluR6 and examine the effects of the peptide on the associations of GluR6 and MLK3 with PSD-95 and the activation of MLK3, JNK3 and c-Jun. Results of reciprocal co-IP and IB showed that pretreatment of the peptide not only diminished the increased interactions of GluR6 with PSD-95 and MLK3 with PSD-95, but also diminished the interaction of GluR6 with MLK3. These results suggested that the peptides could competitively bind to the PDZ1 domain of PSD-95 and suppressed the interaction of GluR6 with PSD-95 and MLK3 in the period of ischaemia/reperfusion. It has been reported that JNK activation contributed to the delayed neuronal cell death induced by cerebral ischaemia. As mentioned before, mice that are deficient in JNK3 are resistant to the hippocampal neurotoxic events related to administration of the glutamate receptor agonist kainic acid. However, JNK1 or JNK2 deficient mice are not resistant to either kainic acid-mediated seizures or neuronal death (Yang et al., 1997). These data suggested that the different JNK isoforms regulate differential responses to neuronal insults with the JNK3 gene being involved in glutamate excitotoxicity, an important mechanism underlining ischaemic death. These data also implicate JNK3 signalling in a brain region that is vulnerable to global ischaemic conditions. Our previous study suggested that activation of JNK3 and one of its upstream molecules, MLK3, which can activate JNK3 through a complex (Whitmarsh et al., 1998) containing JIP1, MKK7 and JNK3, were related to neuronal death induced by the ischaemia/reperfusion (Tian et al., 2003a, b). Current study showed that pretreatment of the peptides could attenuate the activation of MLK3 induced by ischaemia followed by reperfusion. It is interesting to find out that pretreatment of the peptides could only attenuate the second peak level of JNK3 with the 30 min activation unaffected, which was consistent with our previous study indicating DNQX, a selective non-NMDA receptor antagonist, was inefficacious in the prevention of 30 min JNK3 activation. However, NAC, an antioxidant reagent, could effectively inhibit JNK3 activation at 30 min reperfusion, which suggested that 30 min activation of JNK3 was related to the generation of free radicals during the early reperfusion (Tian et al., 2003a). Furthermore, the capability of DNQX in the inhibition of 3d JNK3 activation fits well with our hypothesis that KA receptor subunit GluR6 mediates the activation of JNK3 in the ischaemia followed by reperfusion. Since pretreatment of the peptides could inhibit the activation of MLK3 and JNK3 induced by ischaemia/reperfusion, we inferred that application of the peptides would also rescue hippocampal CA1 neurons from degeneration. Results from our current study showed that the peptides actually had the ability to prevent hippocampal CA1 neurons from degeneration after cerebral ischaemia/reperfusion. Furthermore, neuroprotective role was also observed after application of the peptides 1 h after the ischaemic insult (data not shown). At the same time, the peptides could also protect neurons against apoptosis induced by KA in vitro. Collectively, our results indicate that KA receptor is involved in ischaemic brain injury induced by transient brain ischaemia/reperfusion and the peptides play a neuroprotective role through inhibiting the assembly of GluR6-PSD-95-MLK3 signalling module.

Recently, it has been demonstrated that Bax plays an essential role in inducing apoptosis in response to stress stimuli, as revealed by gene disruption of Bax and of Bax and Bak (Knudson et al., 1995; Lindsten et al., 2000; Wei et al., 2001; Zong et al., 2001). A substantial proportion of Bax is bound to 14-3-3 proteins in the cytosol of healthy cells. In response to stress stimuli, Bax dissociates from 14-3-3 and redistributes to mitochondria (Nomura et al., 2003). After translocation to mitochondria, Bax induces cytochrome c release either by forming a pore by oligomerization in the outer mitochondrial membrane, or by opening other channels. (Shimizu et al., 1999; Saito et al., 2000; Kuwana et al., 2002). Recent studies have also shown that 14-3-3 proteins prevent apoptosis through sequestration of Bax (Samuel et al., 2001; Nomura et al., 2003). However, JNK can promote Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins (Tsuruta et al., 2004). Since Tat-GluR6-9c could inhibit the activation of JNK, we suppose that pretreatment of the peptides should have the ability to inhibit the phosphorylation of 14-3-3 proteins and prevent Bax translocation to mitochondria and attenuate the release of cytochrome c and caspase-3 activation. As a matter of fact, results from western blot and immunohistochemistry show high fidelity to our hypothesis. At the same time, results from TUNEL provided strong evidence that the peptide could protect the hippocampal CA1 neurons from apoptosis. These results suggest that GluR6 mediated signal pathway is involved in neuronal apoptosis induced by ischaemia/reperfusion via non-nuclear pathway, i.e. the mitochondria-dependent apoptosis pathway.

In addition to the non-nuclear pathway, JNK could promote neuronal cell apoptosis also by regulating the activation of some nuclear substrates such as c-Jun. In fact, studies suggested that c-Jun plays an important role in neuronal cell death under in vitro and in vivo conditions (Estus et al., 1994; Ham et al., 1995). Activated JNK phosphorylates the transcription factor c-Jun and leads to increase AP-1 transcription activity to modulate transcription of a number of genes such as Fas ligand (Faris et al., 1998). The increased Fas L can further activate ASK1 through binding to its receptor Fas and in turn, the activated ASK1 phosphorylates JNK. Our results show that pretreatment of Tat-GluR6-9c can diminish the increased phosphorylation of c-Jun and similar results are obtained from immunohistochemistry. Moreover, pretreatment of Tat-GluR6-9c can also significantly diminish the increased expression of Fas L induced by ischaemia and reperfusion. Taken together, these results suggest that GluR6...
Fig. 5 Continued
mediated signal pathway is involved in neuronal apoptosis induced by ischaemia/reperfusion via nuclear pathway.

In recent years, more attention was paid to the Tat-peptides as therapy for ischaemic brain damage. In the recent study, a peptide comprising the nine C-terminal amino acids of NMDA receptor was used to treat stroke by perturbing the interactions between NMDA receptor and PSD-95 (Aarts et al., 2002). Another study showed that a peptide comprising the JNK binding motif (JBD) of JIP-1 and the Tat transporter sequence has an extremely potent protective role in vivo against cerebral ischaemia (Borsello et al., 2003). In general, ionotropic glutamate receptors can control influx or outflux of the cations; on the other hand, it also mediates the intracellular signal transduction via the interaction of the receptors and signal proteins. However, studies indicated that disruption of the NMDA receptor-PSD-95 interaction in hippocampal neurons did not affect the physiological role of the NMDA receptor (Aarts et al., 2002; Lim et al., 2003). In our study, the GluR6 C-terminus containing peptides also show neuroprotection in vivo and in vitro without affecting the currents of KA receptors. On the other hand, the interaction of GluR6 and PSD-95 but not that of NMDA and PSD-95 was affected by Tat-GluR6-9c, which rendered the peptides relative high specificity. Thus, these peptides can be used as non-receptor competitive antagonists to block the glutamate receptor-mediated downstream signalling cascade and subsequently prevent delayed neuron death induced by cerebral ischaemia. In contrast to glutamate receptor antagonists, one distinct advantage of the peptides was blocking the receptor-mediated downstream events without affecting the normal function of the receptors. Second, membrane transduction domain of the HIV-1 was fused to these blocking peptides, rendering them cell permeable, and the unique sequence of these peptides ensured the specific interaction.
with corresponding receptors. It is also interesting to investigate the effect of the neuroprotection by combining these blocking peptides together.

Lack of selective antagonists hampered research on KA receptors for many years. To date, the physiological and pathological function of KA receptors still remains unclear. Recently, with the discovery of selective AMPA receptor and KA receptor subunit GluR5 antagonists (Paternain et al., 1995; Clarke et al., 1997), much progress had been made on understanding the functional properties of KA receptors. Cumulative studies showed that KA receptors were more abundant in CA3 than in CA1 subfields of the hippocampus (Bureau et al., 1999), while studies indicate that degenerating neurons induced by global ischaemia/reperfusion are typically found at the CA1 subfields of the hippocampus. Then, a question raised on how to explain the selective injury in CA1 during ischaemia, when GluR6 is more abundant in CA3. The possible explanation about the discrepancy may be attributed to existence of the anti-apoptotic proteins in CA3/DG region, since cell survival is determined by a balance between survival and death signalling pathways. Our previous study indicated that ERK (Wang et al., 2005), which is considered as anti-apoptotic kinase, are selectively activated in CA3/DG region but not in CA1. Therefore, these anti-apoptotic proteins may inhibit the GluR6-mediated JNK activation and neuronal cell death in CA3 region of the hippocampus. In our present study, pretreatment of the peptide could prevent GluR6-mediated JNK3 activation and rescue the neurons from degeneration in CA1 subfields in response to cerebral ischaemia, which indicated that KA receptor subunit GluR6 participated in the neuronal injury of CA1 subfields in cerebral ischaemia.

Taken together, our results indicate that cerebral ischaemia/reperfusion induced the increased assembly of the GluR6*PSD-95*MLK3 signalling module and subsequently activated MLK3 and JNK3. GluR6-containing KA receptors participated in the neuronal cell death induced by cerebral ischaemia/reperfusion in rat hippocampal CA1 regions. Application of a GluR6 c-terminus containing peptide could suppress the clustering of GluR6 in the postsynaptic regions by competitively binding to the PDZ1 domain of PSD-95 and subsequently inhibit the assembly of the GluR6*PSD-95*MLK3 signalling module, which

Fig. 6 Example of cresyl violet-stained sections of the hippocampi of sham operated rats (A, E) and rats subjected to 15 min of ischaemia followed by 5 days of reperfusion (B, F), and rats subjected to 15 min of ischaemia followed by 5 days of reperfusion with administration of the control peptides (C, G), 100 μg/10 μl of Tat-GluR6-9c 40 min before ischaemia (D, H). Data were obtained from six independent animals and the results of a typical experiment are presented. Boxed areas in left column are shown at higher magnification in right column. A, B, C, D: ×40; E, F, G, H: ×400. Scale bar in D = 200 μm; scale bar in H = 10 μm.

Fig. 7 Effects of Tat-GluR6AA and Tat-GluR6-9c on apoptosis-like cell death induced by KA stimulation. DAPI staining was carried out at 24 h after KA stimulation. Typical apoptotic cells with condensed nucleus are marked by arrow heads. Cells of group of sham control were restored for 24 h. Apoptotic-like cells were expressed as percent of total cells counted in 10 microscopic fields (>400) for DAPI staining. (A) Typical photographs showing DAPI staining of inhibitory effects of Tat-GluR6-9c on KA induced apoptosis-like cell death. Neurons were treated with 50 nM Tat-GluR6AA or Tat-GluR6-9c 1 h before KA stimulation or MK-801 20 min before KA stimulation. (B) Quantitative representations expressed as percentage of total cells counted in 10 microscopic fields (>400) for DAPI staining. \(^a P < 0.05\) versus group of sham, \(^b P < 0.05\) versus group of KA stimulation. (KA: 100 μM KA + 30 μM GYKI 52466; MK-801: 20 μM)
results in declined activation of MLK3, JNK3 and c-Jun. Therefore, the peptides could inhibit the increased expression of Fas-L via the nuclear-pathway and attenuate the increased release of cytochrome c via the mitochondria-dependent non-nuclear-pathway, which ultimately eliminates the activation of caspase-3. Most important, the peptides have a neuroprotective effect on ischaemic brain damage in vivo and on KA-induced excitotcity in vitro, thus, GluR6 c-terminus containing peptide provides a promising therapeutic approach for ischaemic brain injury.

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References

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