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ORIGINAL ARTICLE

Protective functions of taurine against experimental stroke through depressing mitochondria-mediated cell death in rats

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Abstract Taurine, an abundant amino acid in the nervous system, is reported to reduce ischemic brain injury in a dose-dependent manner. This study was designed to investigate whether taurine protected brain against experimental stroke through affecting mitochondria-mediated cell death pathway. Rats were subjected to 2-h ischemia by intraluminal filament, and then reperfused for 22 h. It was confirmed again that taurine (50 mg/kg) administered intravenously 1 h after ischemia markedly improved neurological function and decreased infarct volume at 22 h after reperfusion. In vehicle-treated rats, the levels of intracellular ATP and the levels of cytosolic and mitochondrial Bcl-xL in the penumbra and core were markedly reduced, while the levels of cytosolic Bax in the core and mitochondrial Bax in the penumbra and core were enhanced significantly. There was a decrease in cytochrome *C* in mitochondria and an increase in cytochrome *C* in the cytosol of the penumbra and core. These changes were reversed by taurine. Furthermore, taurine inhibited the activation of calpain and caspase-3, reduced the degradation of αII-spectrin, and attenuated the necrotic and apoptotic cell death in the penumbra and core. These data demonstrated that preserving the mitochondrial function and blocking the mitochondria-mediated cell death pathway may be one mechanism of taurine's action against brain ischemia.

Keywords Taurine · Experimental stroke · Mitochondria · Calpain · Caspase-3

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Abbreviations

ac-DEVD-AFC	*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin
COX IV	Cytochrome *C* oxidase subunit IV isoform
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol bis(2-aminoethyl ether)tetraacetic acid
HE staining	Hematoxylin eosin staining
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-2′-ethanesulfonic acid
MCAo	Middle cerebral artery occlusion
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TTC	2,3,5-Triphenyltetrazolium chlorides
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling
NBT/BCIP	Nitroblue tetrazolium/5-bromo-4-chloro-3-inoloyl-phosphate

Introduction

Mitochondria are not only an important source of ATP, but they also preserve intracellular calcium homeostasis, control of neuronal excitability and synaptic function (Gross et al. 1999; Lipton 1999). It is demonstrated that mitochondria are involved in cell death due to experimental stroke (Sims and Anderson 2002; Nakka et al. 2008). Severe insults of cerebral ischemia render mitochondrial dysfunctional for ATP production. Caspase-dependent apoptosis requires ATP, but a sudden decrease in ATP

levels alternatively induces necrotic cell death. The cell death pathway mediated by mitochondria has been demonstrated in ischemic brain with the release of cytochrome *C* from mitochondria. The release of cytochrome *C* during cerebral ischemia activates the caspase cascade leading to apoptosis, but also impairs the mitochondrial function, consequently resulting in the reduction of ATP synthesis, dysfunction of ion channels, intracellular ion derangements and necrotic cell death.

Bcl-2 family plays crucial roles in the regulation of mitochondria-mediated cell death during experimental stroke (Gross et al. 1999; Lipton 1999; Nakka et al. 2008). Bax and Bcl-xL are two members of Bcl-2 family. Proapoptotic Bax is localized to cytosol. A rapid translocation of cytosol Bax to the mitochondria has been observed after brain ischemia, and the release of cytochrome *C* from mitochondria coincides with the temporal and regional distribution of Bax (Cao et al. 2001; Solaroglu et al. 2006). Conversely, antiapoptotic Bcl-xL, which is localized to the outer mitochondrial membrane, can form heterodimers with proapoptotic proteins, blocking the release of cytochrome *C* (Yang et al. 1997; Finucane et al. 1999). Overexpression of Bcl-xL is neuroprotective against brain ischemia, which is associated with the inhibition of cytochrome *C* release and caspase-3 activation (Finucane et al. 1999; Kilic et al. 2002).

Caspases and calpains are two intracellular cysteine proteases. Caspases are specifically activated in response to apoptotic stimuli, and caspase-3 is believed to be a final killer of apoptosis (Wang 2000). Calpains, activated by calcium and autolytic processing, are primarily involved in necrotic cell death (Wang 2000; Goll et al. 2003). A growing number of reports reveal that calpain can function in apoptotic cell death through processing some apoptosis-regulatory proteins (Bevers and Neumar 2008) and caspase-3 can involve in a typical necrotic death routine through cleaving plasma membrane calcium pump (Schwab et al. 2002). The involvement of calpains and caspase-3 in ischemic cell death has been demonstrated through investigating the protection of their inhibitors and the proteolysis of their substrates (Lipton 1999; Bevers and Neumar 2008; Nakka et al. 2008).

Taurine is the major intracellular free β-amino acid present in most mammalian tissues (Huxtable 1992). It plays an important role in the modulation of neurotransmitter release, calcium homeostasis, osmoregulation, inflammation and neuroprotection (Huxtable 1992; Birdsall 1998; Saransaari and Oja 2000; Schuller-Levis and Park 2004; El Idrissi 2008). In vitro taurine is reported to reduce ischemia-induced caspase-8 and caspase-9 expression in the hypothalamic nuclei (Taranukhin et al. 2008) and attenuate apoptotic neuronal death due to glutamate through decreasing intracellular calcium elevation, depressing calpain activation and enhancing the Bcl-2:Bax ratio (Leon et al. 2009). In vivo taurine is demonstrated to protect the brain against experimental stroke in a dose-dependent manner and taurine at the doses of 5–50 mg/kg has marked protection (Sun and Xu 2008).

With this background knowledge, we hypothesized that taurine could reduce ischemic brain injury through blocking mitochondria-mediated cell death pathway. This study was designed to evaluate the effects of taurine at the dose of 50 mg/kg on the levels of ATP, Bcl-xL and Bax, the release of mitochondrial cytochrome *C*, and the activation of calpain and caspase-3 in the rat model of ischemic stroke.

Materials and methods

Rat model of focal cerebral ischemia

All animal procedures were in accordance with the guidelines for care and use of laboratory animals and were approved by the institutional animal care and use committee. Under chloral hydrate anesthesia (400 mg/kg, i.p.), male adult Sprague–Dawley rats (weighing 315–340 g, Beijing Vital River Experimental Animals Technology Ltd.) were subjected to middle cerebral artery occlusion (MCAo) using an intraluminal filament as described previously (Sun et al. 2009). Reperfusion was accomplished by withdrawing the filament 2 h after MCAo. Sham-operated animals were subjected to the same surgical procedure without MCAo.

Experimental protocols

For evaluating the effects of taurine on neurological deficits and infarct volume, rats were randomly assigned to two groups treated with taurine (Shanghai Chemical Reagents Company; dissolved in normal saline, 50 mg/kg) or vehicle (0.9% saline). In the experiments of ATP levels and caspase-3 activities, Western blot analysis and histopathology, rats were randomly assigned to three groups treated with taurine or vehicle: (1) taurine (50 mg/kg) (2) vehicle (0.9% saline) and (3) sham (0.9% saline). The respective agent (1 ml/kg) was administered intravenously 1 h after MCAo. Neurological deficits, infarct volume and histopathology ($n = 7$ per group) were evaluated after 22 h of reperfusion after 2-h of MCAo (R 22 h). In the experiments of assay of ATP levels and caspase-3 activities, and Western blot analysis, the tissues of penumbra and core in vehicle- or taurine-treated rats were dissected at R 22 h, and regions from the right hemispheres that corresponded to core and penumbra in sham-operated rats were dissected 24 h after operation. The activities of caspase-3, and the levels of

ATP, cytochrome *C*, Bax, Bcl-xL and αII-spectrin were determined ($n = 5$ per group).

Neurological evaluation

Animals were examined for neurological deficits at R 22 h by a investigator blinded to the identity of the groups using a six-point neurological function score: 0, no spontaneous motor activity; 1, spontaneous circling; 2, circling if pulled by tail; 3, lowered resistance to lateral push (and forelimb flexion) without circling; 4, contralateral forelimb flexion; 5, no observable neurological deficit (Schmid-Elsaesser et al. 1998).

Measurement of infarct volume

Rats were anesthetized with chloral hydrate and decapitated at R 22 h. The brains were rapidly removed and sliced into 2 mm-thick slices. The brain slices were stained with 1% 2,3,5-triphenyltetrazolium chlorides (TTC, Sigma Co., St Louis, MO, USA) at 37°C for 15 min in the dark, and then fixed by 4% formaldehyde in phosphate buffered solution. The unstained area of the brain slice was defined as infarction, and the infarct volume was measured as described in our previous report (Sun et al. 2009).

Sample collection and preparation

The tissues of penumbra and core were dissected according to the experimental protocols at 4°C by the method described previously (Ashwal et al. 1998; Sun et al. 2009). For the measurement of intracellular ATP levels, the tissues were weighed and homogenized in 0.5 ml of 0.4 M hyperchloric acid containing 1 mM ethylenediaminetetraacetic acid (EDTA) and then neutralized with 80 µl of 2.5 M $KHCO_3$. Cellular debris was removed by centrifugation at 10,000*g* at 4°C for 10 min. The supernatant was used to determine the intracellular ATP levels (Lowry and Passonneau 1972). For caspase-3 activity assay and Western blot analysis, samples were prepared as described previously (Solaroglu et al. 2006). Briefly, the tissues were homogenized in five volumes of homogenization buffer [20 mM *N*-2-hydroxyethylpiperazine-*N*′-2′-ethanesulfonic acid (HEPES), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM ethyleneglycol bis(2-aminoethyl ether)tetraacetic acid (EGTA), 250 mM sucrose, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol (DTT) and 10 µg/ml of each of aprotinin, pepstatin A and leupeptin, pH 7.9]. Samples were centrifuged at 750*g* at 4°C for 15 min. The pellet was discarded. The supernatant, containing the cytosolic/mitochondrial protein was further centrifuged at 16,000*g* for 30 min at 4°C to separate supernatant from pellet. The supernatant was used as the cytosolic fraction and the pellet as the mitochondrial fraction after resuspension in 100 µl of homogenization buffer. The protein concentrations in cytosolic and mitochondrial fractions were determined by the method of Bradford (1976).

Measurement of intracellular ATP levels

A 50 µl sample was added to assay the solution containing 0.4 mM glucose 50 µl, 2 mM nicotinamide-adenine dinucleotide phosphate 50 µl, 40 mM $MgCl_2$ 50 µl, 20 mM DTT 50 µl, 1,670 µl tris(hydroxymethyl)aminomethane (pH 7.4), 27 U/ml hexokinase 20 µl and 7 U/ml glucose-6-phosphate dehydrogenase 10 µl. The samples were incubated at 37°C for 15 min. The fluorescence intensity was measured with a spectrofluorometer set at 350 nm excitation wavelength and at 450 nm emission wavelength (Hitachi Co., Japan) (Lowry and Passonneau 1972). The results were expressed as percent of the levels in sham-operated rats.

Caspase-3 activity assay

The caspase-3 activity was determined by use of *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (ac-DEVD-AFC) (Calbiochem, San Diego, CA, USA), a fluorescent substrate to be used as a susceptible fluorescent substrate for caspase-3. This assay is based on detecting the cleavage of substrate DEVD-AFC, as described previously (Benchoua et al. 2001). Briefly, 15 µl sample was diluted in caspase-3 assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM DTT) and incubated at 37°C for 2 h and added to 15 µl of a 2 mM solution of ac-DEVD-AFC. The amounts of released 7-amino-4-trifluoromethylcoumarin were measured with a fluorescent spectrophotometer with excitation at 400 nm and emission at 505 nm. The results were expressed as percent of the levels in sham-operated rats.

Western blot analysis

The proteins in the sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) described in our previous paper (Sun et al. 2009). As much as 30 µg of proteins was separated by SDS-PAGE, and molecular weight markers (New England Biolabs Inc., Ipswich, MA, USA) were loaded on each gel for protein band identification. The proteins on the gel were subsequently transferred onto a polyvinylidene difluoride membrane using a semidry transfer apparatus. The membrane was then probed with antibody reactive to Bax, Bcl-xL, cytochrome *C* or αII-spectrin (1:100; Santa Cruz

Biotechnology, CA, USA) at 4°C overnight and subsequently incubated with horseradish peroxidase-conjugated secondary antibody for 1.5 h at room temperature. Finally, the color reaction was observed by incubation of the membrane with nitroblue tetrazolium/5-bromo-4-chloro-3-inoloyl-phosphate (NBT/BCIP) (Amresco, Solon, OH, USA), and the integrated optical densities of the protein bands, detected by Western blot analysis, were analyzed by gel image analyzer (AlphaImagerTM 2200, Aalpha Innotech Co., USA). β-actin (1:400; ProteinTech Group, Inc., Chicago, IL, USA) and cytochrome *C* oxidase subunit IV isoform (COX IV) (1:100; Santa Cruz Biotechnology) were used as internal controls for the cytosolic and mitochondrial fractions, respectively. The results were expressed as percentage of the levels in sham-operated rats.

Histopathological analysis

The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining was used to analyze the apoptotic cells coupled with the identification of relevant morphological changes of apoptotic cell death including membrane integrity, cell shrinkage, cytoplasmic and nuclear condensations and apoptotic bodies, notwithstanding that this technique can detect the DNA fragmentation in necrotic and apoptotic cells under pathological conditions (Graham and Chen 2001; Sims and Anderson 2002). Hematoxylin eosin (HE) and cresyl violet staining were used to investigate necrotic cell death. Animals were anesthetized with chloral hydrate and transcardially perfused with heparinized normal saline followed by 4% paraformaldehyde 24 h after MCAo. Brains were removed, fixed, embedded in paraffin and the 8 μm-thick coronal sections through the anterior commissure were collected. TUNEL, HE and cresyl violet staining were performed following the procedures described previously (Sun and Xu 2008; Sun et al. 2009). The sections were examined with light microscopy and pictures were taken with a digital camera. The apoptotic neurons display positive TUNEL staining, membrane integrity, cell shrinkage, cytoplasmic and nuclear condensations and apoptotic bodies in TUNEL-stained sections, and the necrotic neurons show disappearance of Nissl body in cytoplasma, chromatolysis, nuclear pyknosis, eosinophilic cytoplasm (red neuron) or lack cellular structure (ghost neuron) in HE- and cresyl violet-stained sections (Sun et al. 2009). The apoptotic and necrotic cells in the penumbra and core were evaluated by a five-point score that was described previously: 0, normal; 1, damaged neurons were <25%; 2, damaged neurons were 25–50%; 3, damaged neurons were 50–75%; and 4, damaged neurons were >75%.

Data expression and statistical analysis

Data are presented as mean ± SEM. Comparisons between groups were statistically evaluated by Student's *t* test (infarct volume) or one-way ANOVA with a post hoc Fisher's test (the activities of caspase-3, and the levels of ATP, Bax, Bcl-xL, cytochrome *C* and αII-spectrin). Neurological deficits, TUNEL-positive staining score and the necrotic cell death score were analyzed with a nonparametric Mann–Whitney *U* test. A probability of <0.05 was considered to be statistically significant.

Results

Effects of taurine on neurological deficits and infarct volume

Before ischemia, neurological scores were normal (score = 5) in all animals. The vehicle-treated rats showed significant neurological deficits at R 22 h. Treatment with taurine markedly reduced the neurological deficits (Fig. 1a; $P < 0.01$ vs. vehicle-treated rats). Representative coronal brain sections from vehicle- and taurine-treated rats stained with 1% TTC at R 22 h are shown in Fig. 1b. 2-h ischemia following 22-h reperfusion resulted in an infarct of 175 ± 13 mm^3 in vehicle-treated rats (Fig. 1c). The infarct volume was markedly decreased in taurine-treated rats versus vehicle-treated rats ($P < 0.01$).

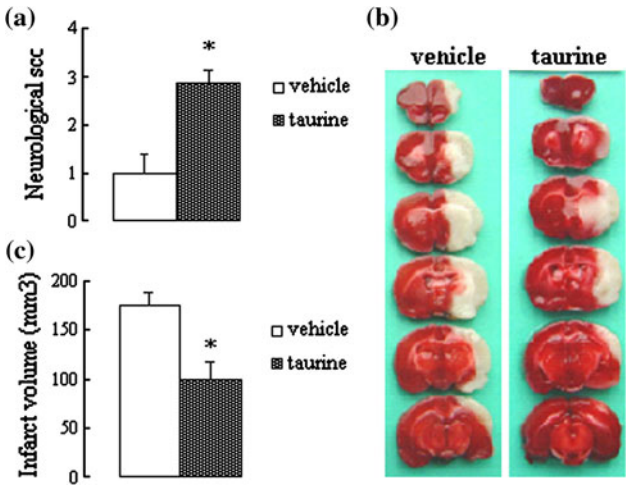

Fig. 1 Effects of taurine on the neurological score and infarct volume at 22 h of reperfusion after 2-h of focal cerebral ischemia. Vehicle or taurine was injected intravenously 1 h after ischemia. Data were presented as mean ± SEM. $n = 7$. **a** Effects of taurine on neurological deficits (*$P < 0.01$ vs. vehicle). **b** The *infarct zone* was displayed by TTC staining in vehicle- or taurine-treated rats. **c** The *bar* graph presented the infarct volumes from TTC staining in vehicle- or taurine-treated rats (*$P < 0.01$ vs. vehicle)

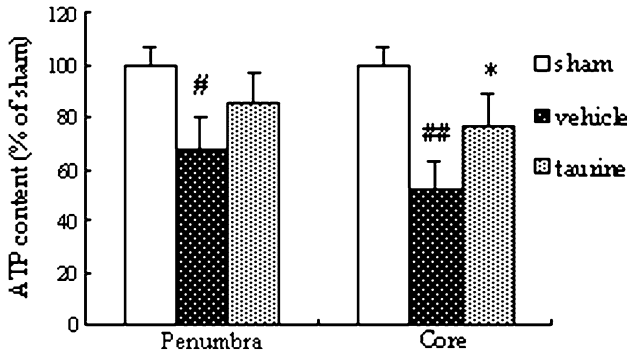

Fig. 2 Effects of taurine on the intracellular ATP levels in the penumbra and core at 22 h of reperfusion after 2 h of focal cerebral ischemia. Vehicle or taurine was injected intravenously 1 h after ischemia. The results were expressed as percentage of the levels in sham-operated rats (mean ± SEM. $n = 5$. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. sham-operated rats.$^{*}P < 0.05$ vs. vehicle-treated rats)

Effects of taurine on intracellular ATP levels

The marked reduction of the levels of intracellular ATP in the penumbra and core at R 22 h in vehicle-treated rats was found (Fig. 2; $P < 0.05$ and 0.01 vs. sham-operated rat). Taurine treatment enhanced the levels of intracellular ATP in the core significantly versus vehicle-treated rats ($P < 0.05$). It also increased the levels of intracellular ATP in penumbra, but did not reach statistical significance.

Effects of taurine on caspase-3 activities

The caspase-3 activities in cytosolic fractions were measured through determining the cleavage of fluorescent substrate ac-DEVD-AFC. As illustrated in Fig. 3, the caspase-3 activities in penumbra and core at R 22 h increased significantly (both $P < 0.01$ vs. sham-operated rats), and taurine treatment markedly reduced the caspase-3 activities in the penumbra and core ($P < 0.01$ and 0.05 vs. vehicle-treated rats, respectively).

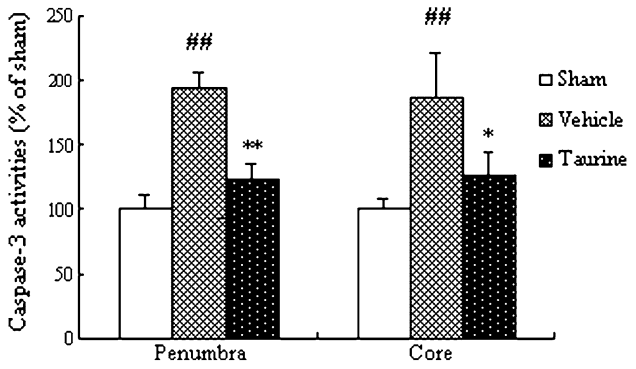

Fig. 3 Effects of taurine on caspase-3 activities in cytosolic fractions in the penumbra and core at 22 h of reperfusion after 2 h of focal cerebral ischemia. Vehicle or taurine was injected intravenously 1 h after ischemia. The results were expressed as percentage of the levels in sham-operated rats (mean ± SEM. $n = 5$. $^{\#\#}P < 0.01$ vs. sham. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle)

Effects of taurine on calpain activation

In the present study, the calpain activation in the penumbra and core was assayed by examining the degradation of a well-characterized calpain substrate, αII-spectrin. Cleavage of endogenous αII-spectrin (240 kDa) into 145 kDa fragment is indicative of the activation of calpain. As illustrated in Fig. 4, the levels of αII-spectrin in the penumbra and core following experimental stroke were decreased significantly ($P < 0.05$ and 0.01, respectively), and the levels of 145 kDa fragment of αII-spectrin in the penumbra

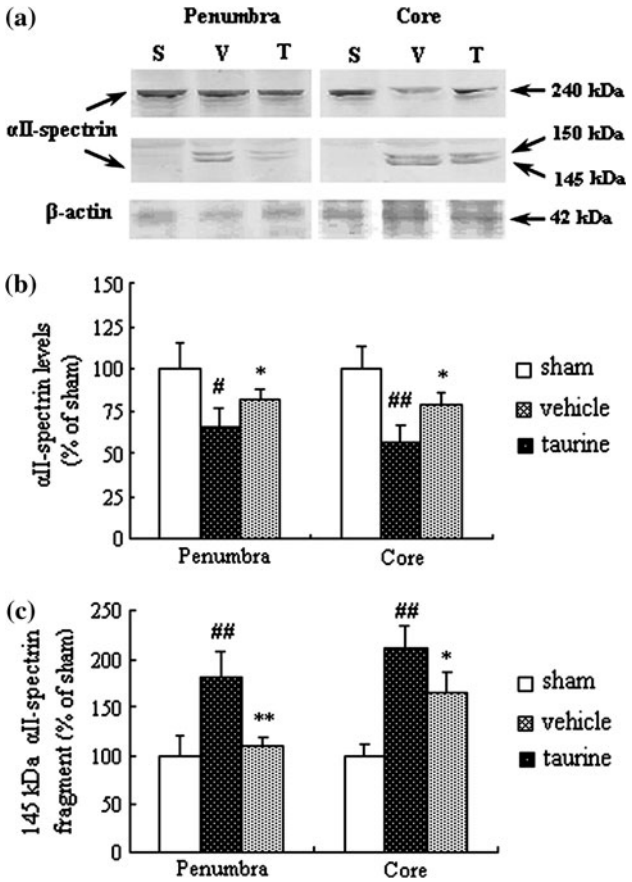

Fig. 4 Effects of taurine on the levels of 240 kDa αII-spectrin and 145 kDa αII-spectrin fragment in the penumbra and core at 22 h of reperfusion after 2 h of focal cerebral ischemia. Vehicle or taurine was injected intravenously 1 h after ischemia. **a** Western blot analysis using αII-spectrin antibody. *S* sham, *V* vehicle, *T* taurine (**b**, **c**) The *bar* graphs reflected the densitometric data of 240 kDa αII-spectrin and 145 kDa αII-spectrin fragment from the experiment of αII-spectrin Western blot, respectively. The results were expressed as percentage of the levels in sham-operated rats (mean ± SEM. $n = 5$. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. sham. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle)

and core were increased (both $P < 0.01$). Treatment with taurine markedly enhanced the levels of αII-spectrin in the penumbra and core (both $P < 0.05$ vs. vehicle-treated rats), and reduced the levels of 145 kDa fragment of αII-spectrin in the penumbra and core ($P < 0.01$ and 0.05 vs. vehicle-treated rats, respectively). These data indicated the inhibition of αII-spectrin degradation and calpain activation by taurine after experimental stroke.

Effects of taurine on the levels of Bax, Bcl-xL and cytochrome *C*

For analyzing the influence of taurine on the mitochondria-mediated cell death pathway, we investigated the effects of taurine on the levels of Bax, Bcl-xL and cytochrome *C* in cytosolic and mitochondrial fractions at R 22 h, and the results are shown in Fig. 5. The levels of Bax in vehicle-

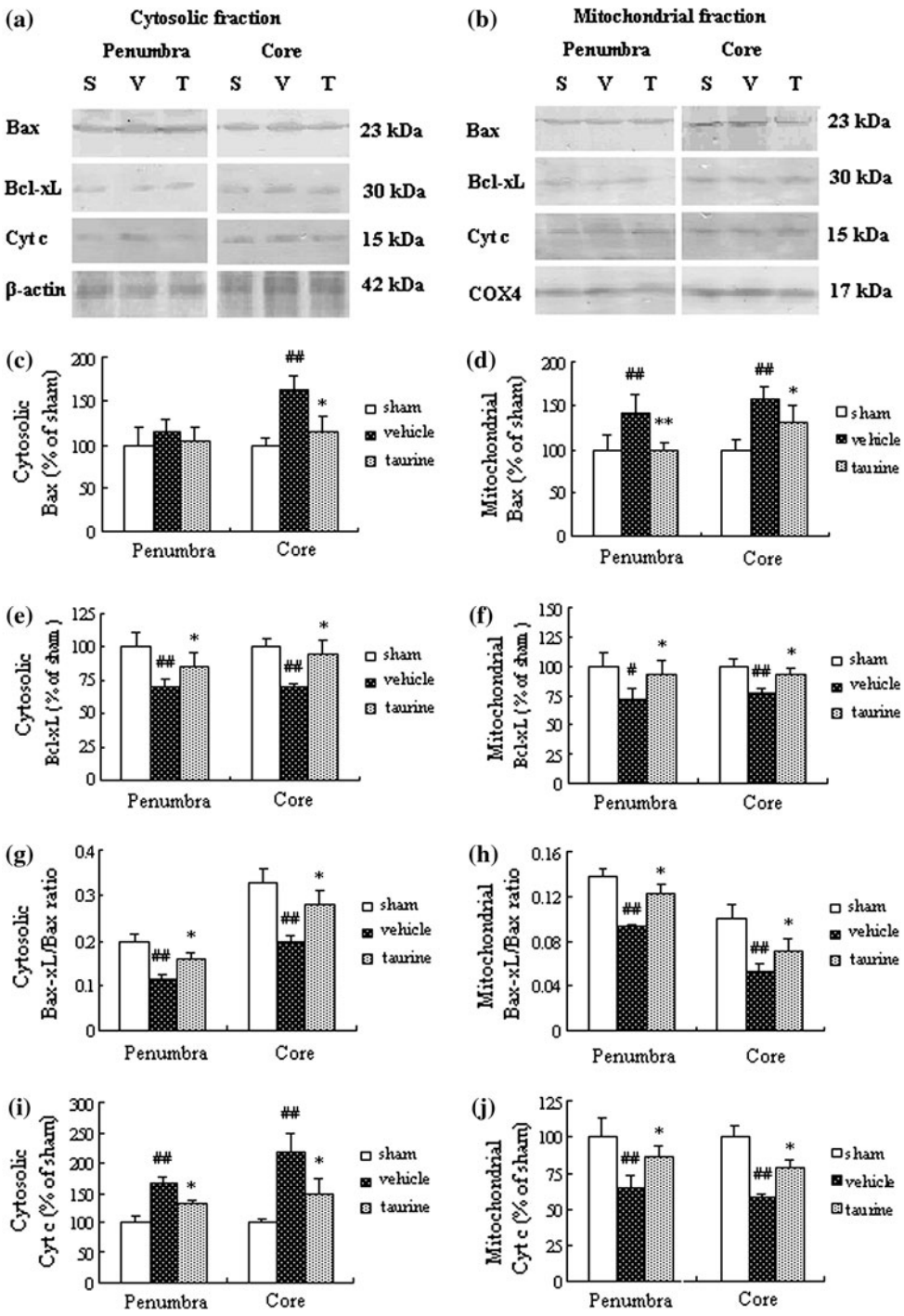

Fig. 5 Effects of taurine on the levels of Bax, Bcl-xL and cytochrome *C* in cytosolic and mitochondrial fractions in the penumbra and core at 22 h of reperfusion after 2 h of focal cerebral ischemia. Vehicle or taurine was injected intravenously 1 h after ischemia. (**a** and **b**) Western blot analysis using Bax, Bcl-xL or cytochrome *C* antibody in cytosolic and mitochondrial fractions, respectively. *S* sham, *V* vehicle, *T* taurine, *Cyt c* cytochrome *C*. (**c**, **d**) (**e**, **f**), and (**i**, **j**) The *bar* graphs reflected the densitometric data from the experiment of Bax, Bcl-xL and cytochrome *C* Western blot in cytosolic and mitochondrial fractions, respectively (the results were expressed as percentage of the levels in sham-operated rats). (**g**, **h**) The *bar* graph reflected the ratio of Bcl-xL/Bax in cytosolic and mitochondrial fractions, respectively. All data were expressed as mean ± SEM. $n = 5$. **b** $^{\#\#}P < 0.01$ vs. sham. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle

treated rats in the cytosolic fraction in the core and in the mitochondrial fractions in the penumbra and core were increased significantly (Fig. 5a, b, c and d; all $P < 0.01$ vs. sham-operated rats), while the levels of Bax in vehicle-treated rats in the cytosolic fraction in the penumbra were increased without statistical significance. In contrast, the levels of Bcl-xL in the cytosolic and mitochondrial fractions in the penumbra and core were markedly reduced versus sham-operated rats (Fig. 5a, b, e and f; $P < 0.01$, 0.01, 0.05 and 0.01, respectively). Ischemia-induced changes of Bcl-xL and Bax levels resulted in a significant reduction in the ratio of Bcl-xL:Bax in the cytosolic and mitochondrial fractions in the penumbra and core (Fig. 5g, h; all $P < 0.01$ vs. sham-operated rats). Also, the levels of cytochrome *C* in cytosolic fractions in the penumbra and core were markedly increased (Fig. 5i; both $P < 0.01$ vs. sham-operated rats), while that in the mitochondrial fraction in the penumbra and core were reduced significantly (Fig. 5j; both $P < 0.01$ vs. sham-operated rats). Compared with vehicle-treated rats, treatment with taurine markedly reduced the levels of Bax in the cytosolic fraction in the core and in mitochondrial fractions in the penumbra and core ($P < 0.05$, 0.01, and 0.05, respectively), and enhanced the levels of Bcl-xL and the ratio of Bcl-xL:Bax in the cytosolic and mitochondrial fractions in the penumbra and core (Bcl-xL or the ratio of Bcl-xL:Bax: all $P < 0.05$). Taurine reduced the levels of cytochrome *C* in the cytosolic fraction and enhanced the levels of cytochrome *C* in the mitochondrial fraction in the penumbra and core (all $P < 0.05$). It also reduced the levels of Bax in the cytosolic fraction in the penumbra, but did not reach statistical significance.

Effect of taurine on DNA fragmentation and cell death

The anatomical distribution of the penumbra and core after experimental stroke is shown in Fig. 6a (Ashwal et al. 1998). HE and cresyl violet staining were used to

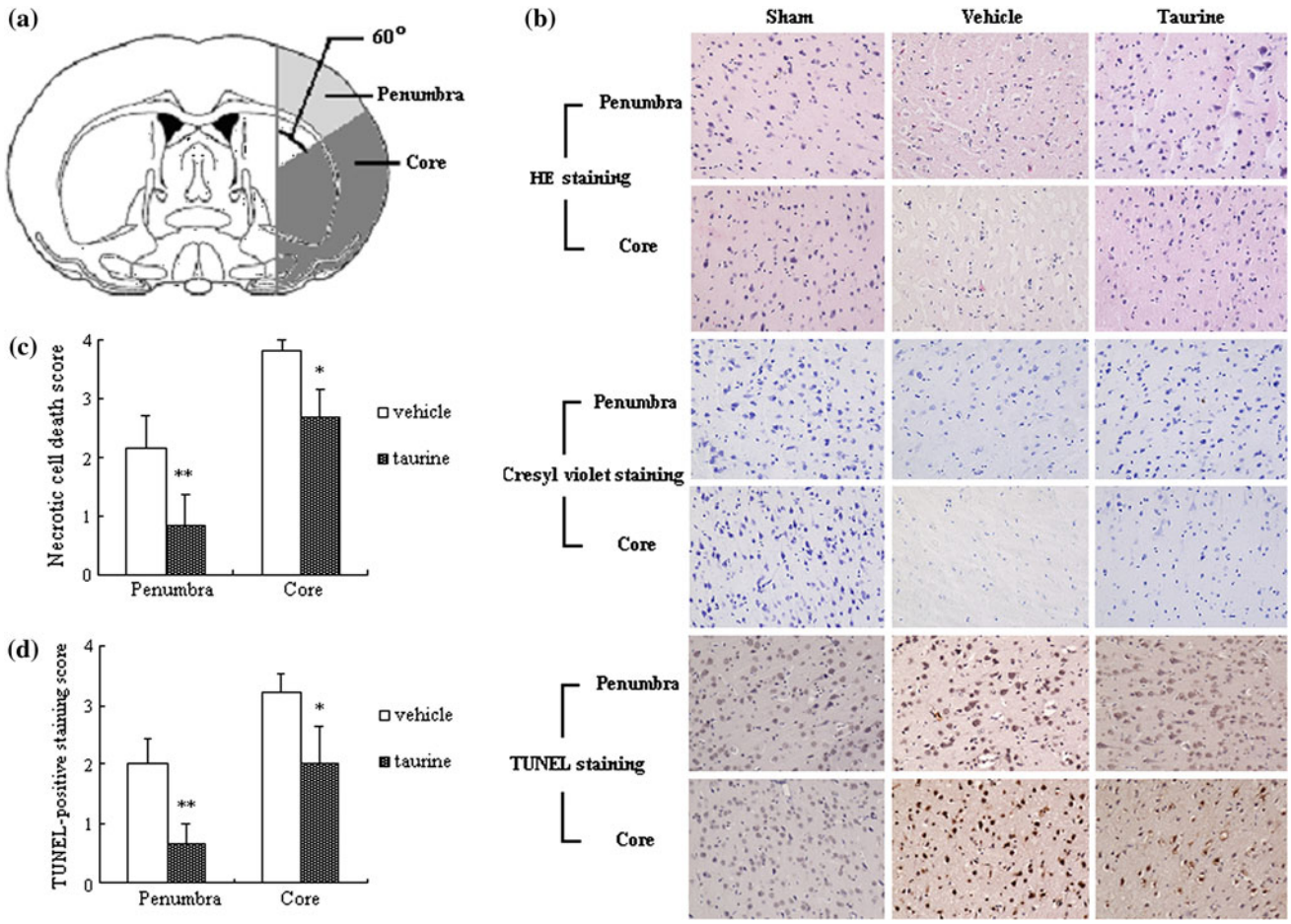

Fig. 6 Effects of taurine on the necrotic and apoptotic cell death in the penumbra and core at 22 h of reperfusion after 2 h of focal cerebral ischemia. Vehicle or taurine was injected intravenously 1 h after ischemia. **a** Anatomical distribution of penumbra and core. **b** Representative photographs stained by HE, Cresyl violet and TUNEL (original magnification ×200). (**c**, **d**): The *bar* graphs reflected the necrotic cell death score and the TUNEL-positive cell score in the penumbra and core in each group, respectively (mean ± SEM. $n = 7$. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle)

investigate the morphology of necrotic cells in the penumbra and core after experimental stroke, and the results are illustrated in Fig. 6b, c. In sham-operated rats, neurons in the cortex showed intact morphology. In ischemic core in vehicle-treated rats, most neurons showed morphological characteristics of necrosis. Although it was not as severe as in the core, necrotic changes were also observed in some cells in the penumbra in vehicle-treated rats. Compared with vehicle-treated rats, the necrotic cell death scores in the penumbra and core were reduced significantly in taurine-treated rats ($P < 0.01$ and 0.05, respectively).

The DNA fragmentation was determined by TUNEL staining, and the results are illustrated in Fig. 6b, d. These TUNEL staining positive cells were densely labeled in the nuclei and showed morphologic signs of apoptosis. A large number of TUNEL-staining positive cells occurred in the penumbra and core of vehicle-treated rats, while it was not observed in sham-operated rats. Treatment with taurine significantly reduced the TUNEL-staining positive score in the penumbra and core ($P < 0.01$ and 0.05 vs. vehicle-treated rats, respectively).

Discussion

We have reported the dose-dependent protection of taurine; suppression of the over-activation of calpain- and caspase-3 in penumbra may be one mechanism of taurine against ischemic stroke in rats (Sun and Xu 2008). The main purpose of this study was to investigate the effects of taurine at the dose of 50 mg/kg on the mitochondria-mediated cell death pathway in the penumbra and core after experimental stroke. It is found that treatment with taurine enhances the intracellular ATP, down-regulates Bax, up-regulates Bcl-xL, reduces the release of cytochrome *C* from mitochondria, suppresses the activation of calpain and caspase-3, attenuates the apoptotic and necrotic cell death in the penumbra and core, and decreases the infarct volume after experimental stroke. These data confirmed the neuroprotection of taurine and suggest that blocking mitochondria-mediated cell death pathway in the penumbra and core may be one of the mechanisms of taurine against experimental stroke.

Taurine is a key regulator of intracellular homeostasis or enantiostasis (Huxtable 1992; Michalk et al. 1996). During experimental stroke, the level of taurine in extracellular fluid increases and remains elevated throughout the subsequent reperfusion phase (Lo et al. 1998). The increases in the extracellular taurine levels under brain ischemia may constitute an important endogenous protective mechanism against neuronal damage (Saransaari and Oja 2000). However, the depletion of intracellular taurine may result in a disruption of intracellular homeostasis, leading to neuronal damage (Huxtable 1992; Michalk et al. 1996). Thus, the release of taurine may be an obligatory self-protective mechanism under ischemic stress. During cerebral ischemia, taurine may exert its neuroprotective function through both extracellular and intracellular mechanisms. The extracellular action involved in the inhibition of calcium influx is attributed to the suppression of taurine on glutamate-mediated depolarization through opening the chloride channel. The intracellular action of taurine may be related to its protection of the mitochondrial function by preventing mitochondrial dysfunction from calcium overload (Huxtable 1992; Foos and Wu 2002; El Idrissi and Trenkner 2004; El Idrissi 2008). Other functions of taurine, such as anti-oxidation, anti-inflammation and osmoregulation, are also contributed to its neuroprotective action (Huxtable 1992; Schuller-Levis and Park 2004). Therefore, it is reasonable that exogenous administration of adequate amount of taurine after brain ischemia may enhance the levels of extracellular taurine and reduce the release of taurine, thus consequently contributing to the recovery of intracellular homeostasis and the reduction of ischemic damage through both extracellular and intracellular mechanisms.

As a neuroprotective agent, taurine must pass through the blood–brain barrier and enter into the brain under neuropathological conditions. Some researchers have reported the increases in radioactive taurine in the brain after systemic administration of radio-labeled taurine (Urquhart et al. 1974; Pasantes-Morales and Arzate 1981) and the linear increase of taurine concentration in rat brain after intraperitoneal injection (Lallemand and de Witte 2004). In vitro, a Na^+ and Cl^- gradient-dependent transport system for taurine is identified in both the luminal and the antiluminal membranes of bovine brain capillary endothelial cells, and the carrier-mediated transport found by in vitro experiments is confirmed to function for the translocation of the taurine molecule from the vascular space into the brain (Tamai et al. 1995). Moreover, taurine has been used with varying degrees of success in the clinical therapy of epilepsy and other seizure disorders (Birdsall 1998). These data provide convincing evidence that taurine can cross blood–brain barrier and reach the ischemic area to exert its cytoprotection when it is administered intravenously after brain ischemia.

It is believed that intracellular calcium overload due to brain ischemia initiates a series of intracellular events that impact the development of tissue damage profoundly, such as impaired oxidative phosphorylation, release of apoptogenic proteins, generation of reactive oxygen species and activation of degradative enzymes (e.g., calpains, phospholipases and endonucleases), leading to necrotic and apoptotic cell death (Lipton 1999; Nakka et al. 2008). A number of reports have demonstrated the regulation of

taurine on intracellular calcium homeostasis through enhancing the mitochondrial function, reducing the release of calcium from intracellular storage pools, increasing the capacity of mitochondria in sequestering calcium, inhibiting the reversal of sodium/calcium exchangers and affecting the calcium influx (Foos and Wu 2002; El Idrissi and Trenkner 2004; El Idrissi 2008). These data suggest that inhibiting intracellular calcium overload may be essential for the protection of taurine against experimental stroke. It is logical that taurine may block calpain- or caspase-3-mediated cell death due to experimental stroke through preserving mitochondrial function and maintaining intracellular calcium homeostasis, as the increases in intracellular calcium can directly activate calpain (Goll et al. 2003) and induce caspase-3-like activity by regulating the release of mitochondrial cytochrome *C* (Juin et al. 1998). This hypothesis has been demonstrated by our results, although the effect of taurine on intracellular calcium has not been directly investigated in this study.

Calpain has been shown to have a variety of substrates, including structural proteins, calcium-regulatory proteins and signaling proteins (Bevers and Neumar 2008). We are interested in Bcl-xL and Bax, as they are substrates for calpain and involved in the regulation of cell death (Bevers and Neumar 2008). During brain ischemia, Bax expression is increased and translocated to mitochondria from cytosol, while Bcl-xL is decreased (Cao et al. 2001; Ferrer and Planas 2003; Solaroglu et al. 2006) and overexpression of Bcl-xL is neuroprotective (Kilic et al. 2002). These data indicate that the changes of Bax and Bcl-xL during brain ischemia contribute to ischemic cell death. Calpain can cleave the loop region of Bcl-xL, converting it to a proapoptotic molecule (Nakagawa and Yuan 2000). On the contrary, calpain cleavage of Bax in the mitochondrial fraction has been found to cause an increase in 18 kDa Bax levels; it is the active form capable of forming homodimers and possesses more potent cytotoxicity than Bax (Gao and Dou 2000). Bcl-xL inhibits Bax homodimerization by forming Bcl-xL/Bax heterodimers. Imbalances between Bcl-xL and Bax lead to the formation of Bax homodimers, subsequently resulting in mitochondrial permeability transition, cytochrome *C* release, and caspase-9 and caspase-3 activation. Ischemia-induced calpain activation may therefore be responsible for the decrease in Bcl-xL levels and the increase in the Bax levels, consequently leading to the reduction of Bcl-xL/Bax ratios, the activation of caspase cascade and ischemic cell death. Our results suggest that ischemia-induced activation of calpain directly promotes the caspase cascade by its effects on Bcl-xL/Bax ratios. Taurine inhibition of ischemia-induced activation of calpain prevents the decreases in the Bcl-xL/Bax ratios, thus blocking the mitochondria-mediated cell death after experimental stroke.

Traditionally, cell death is separated into categories: apoptosis and necrosis. Apoptosis is an energy-dependent and regulated active process of cell death, while necrosis is thought to be a passive process (Harwood et al. 2005; Martin, 2010). In recent years, increasing evidences show that neuronal degeneration in brain exists as a continuum between apoptosis and necrosis (Martin 2010). This morphological continuum is defined as the occurrence of classic apoptosis and necrosis at opposite ends of the spectrum of cell death with many possible variant forms of cell death residing between the classic endpoints, and the mode of cell death is determined by the energy status under a stress or insult (Nicotera et al. 1998; Martin 2010). The interrelationship between apoptosis and necrosis is highlighted by the fact that necrotic cell death can be mediated by part of the apoptotic machinery, and that both death modes can share a common pathway (Kim et al. 2003; Harwood et al. 2005; Martin 2010). For example, it has been well established that Bcl-2 family of proteins is involved in the regulation of apoptotic and necrotic cell death (Tsujimoto and Shimizu 2000; Choi et al. 2001; Moubarak et al. 2007), and intracellular calcium overload due to neurotoxicity can induce mitochondrial dysfunction, leading to necrotic and apoptotic cell death (Gross et al. 1999; Sims and Anderson 2002; Nakka et al. 2008; Martin 2010). A growing body of literature has emerged, indicating the central roles of caspases and calpains in apoptotic and necrotic cell death (Schwab et al. 2002; Harwood et al. 2005; Bevers and Neumar 2008). Calpain can function in apoptotic cell death through processing some apoptosis-regulatory proteins, such as apoptosis-activating factor 1, caspase-9, Bcl-xL, Bax and p53, and caspase-3 is reported to involve in a typical necrotic death routine through cleaving plasma membrane calcium pump.

It is well known that a lesion due to stroke usually consists of core and penumbra. The designation of these core and penumbral regions is based on the thresholds of the cerebral blood flow, biochemical changes and the studies of pharmacology and histopathology, and experimental stroke models show a gradient in the severity of hypoperfusion and in the energy failure from core to penumbra (Ginsberg 1997; Lipton 1999; Graham and Chen 2001). When ischemia is provoked by MCAo, primary necrotic cell death appears rapidly in the core. In addition to suffering from very suboptimal perfusion levels, cells in the penumbra are subjected to deleterious factors produced by neighboring cells in the core, including inflammatory mediators, reactive oxygen species, glutamate, etc. These factors can determine the destiny of neurons in the penumbra and the expansion of infarct lesion, depending on the status of collateral blood flow, ATP level and/or appropriate therapeutic approach. During cerebral ischemia, increased Bax protein, reduced Bcl-2 or Bcl-x,

activated caspases and TUNEL-positive neurons with apoptotic characteristics can be detected in ischemic penumbra and core (Krajewski et al. 1995; Matsushita et al. 1998; Benchoua et al. 2001; Elibol et al. 2001), and we have reported the cross talk between calpain and caspase-3 in the penumbra and core during experimental stroke (Sun et al. 2008). Therefore, it is reasonable that some apoptotic proteins, including Bcl-2 family and caspases, are involved in cell death in the penumbra and core. In the penumbra, caspase cascades are activated, and apoptosis can fully develop because of residual blood supply. In contrast, the apoptotic pathway is initiated in the core in the early stage of ischemia, but the apoptotic process may be blocked due to the depletion of energy, which may cause a shift toward secondary necrosis from apoptosis (Nakka et al. 2008; Nicotera et al. 1998; Martin 2010). The dead neurons in the core may show the characteristics of apoptosis and necrosis. This study demonstrates that taurine reduces ischemic brain injury through modulating the actions of Bcl-2 family proteins and mitochondria, subsequently inhibiting the activation of calpain and caspase-3, depressing apoptotic and necrotic cell death and finally blocking the expansion of ischemic lesion.

Conclusions

This study demonstrates the protective functions of taurine against experimental stroke through preserving the mitochondrial functions and blocking the mitochondria-mediated cell death pathway in the penumbra and core. These data provide evidences to support the hypothesis that exogenous administration of taurine may reduce ischemic brain damage through modulating intracellular homeostasis. Further studies are needed to elucidate the detailed mechanism by which taurine maintains the mitochondrial function during experimental stroke, which is beneficial to understanding the regulation of taurine on intracellular homeostasis.

Conflict of interest The authors declare that they have no conflict of interest.

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