Free Radical Biology & Medicine 48 (2010) 597–608

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Stabilization of mitochondrial function by tetramethylpyrazine protects against kainate-induced oxidative lesions in the rat hippocampus

Shu-Yan Li [a,*], Yu-Hong Jia [a,1], Wen-Ge Sun [a,2], Yuan Tang [a], Guo-Shun An [a], Ju-Hua Ni [a], Hong-Ti Jia [a,b]

[a] Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Beijing 100191, People's Republic of China
[b] Department of Biochemistry and Molecular Biology, Capital Medical University, Beijing 100069, People's Republic of China

ARTICLE INFO

Article history:
Received 22 July 2009
Revised 4 November 2009
Accepted 7 December 2009
Available online 23 December 2009

Keywords:
Tetramethylpyrazine
Neuroprotection
Kainate
Reactive oxygen species
Mitochondrial dysfunction
Free radicals

ABSTRACT

Mitochondria are critical regulators of cell death, a key feature of neurodegeneration. Reactive oxygen species (ROS) are crucial to Ca^{2+}-mediated effects of glutamate receptor activation leading to neuronal degeneration. Tetramethylpyrazine (TMP) is a principal ingredient of *Ligusticum wallichi* Franchat (a Chinese herb), used for treatment of cardiovascular and cerebrovascular ischemic diseases. However, its protection against oxidative brain injury associated with excessive activation of glutamate receptors is unknown. In this study, we demonstrate TMP neuroprotection against kainate-induced excitotoxicity in vitro and in vivo. We found that TMP could partly alleviate kainate-induced status epilepticus in rats and prevented and rescued neuronal loss in the hippocampal CA3 but not the CA1 region. The partial prevention and rescue of neuronal loss by TMP were attributable to the preservation of the structural and functional integrity of mitochondria, evidenced by maintaining the mitochondrial membrane potential, ATP production, and complex I and III activities. Stabilization of mitochondrial function was linked to the observation that TMP could function as a reductant/antioxidant to quench ROS, block lipid peroxidation, and protect enzymatic antioxidants such as glutathione peroxidase and glutathione reductase. These results suggest that TMP may protect against oxidative brain injury by stabilization of mitochondrial function through quenching of ROS.

Oxidative stress is a causal, or at least an ancillary, factor in the neuropathology of several adult neurodegenerative disorders and in stroke, trauma, and seizures. Glutamate receptors mediate excitatory neurotransmission, and excessive or persistent activation of glutamate receptors may cause neuronal degeneration through Ca^{2+}-mediated effects that may induce oxidative stress in a number of pathways [1,2]. In the models of excitotoxicity and seizures, kainate, an exogenous glutamate analogue, increases production of reactive oxygen species (ROS). ROS can damage a variety of biomolecules such as lipids, proteins, carbohydrates, and nucleic acids, leading to various types of cellular dysfunction. Of the cellular dysfunctions, mitochondrial dysfunction has a causal role in all major neurodegenerative diseases [3].

Apoptosis is implicated in neurodegenerative disorders such as Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, and Huntington disease [4]. Eliciting a rise in intracellular free Ca^{2+} is believed to play a particularly important role in glutamate-induced neuronal death [5]. The abnormal influx of intracellular Ca^{2+} activates a number of proteases, phospholipases, and endonucleases by generating ROS that attack the cellular membrane [6,7] and induce apoptosis [5,6]. The levels of Ca^{2+} and ROS in the cells are regulated by mitochondria and are closely linked to cell survival and death. Cell survival under oxidative stress depends on the levels of intracellular ROS, whose formation is closely related to the balance between oxidants and antioxidants [8,9]. Disruption of the balance, for instance, depletion of glutathione (GSH), may result in increased ROS, promoting cellular damage [9]. By contrast, ROS-quenching agents can effectively protect neurons against excitotoxicity [2].

2,3,5,6-Tetramethylpyrazine (TMP; also known as ligustrazine) is a principal ingredient of *Ligusticum wallichi* Franchat (Chuan Xiong), a promising traditional Chinese herb that is widely used for treatment of patients with cardiovascular diseases and cerebrovascular ischemic diseases in China and Japan [10–12]. Animal experiments have shown that TMP suppresses epilepsy discharge induced by pentylenetetrazol [13] and protects against spinal cord ischemia [14]. The study of mechanisms reveals that TMP may decrease platelet aggregation, inhibit thrombus formation, and improve brain microcirculation in rats [15]. Recent studies have demonstrated that alleviation of the

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CTZ, cyclothiazide; D-AP5, D-2-amino-5-phosphonopentanoic acid; DHE, dihydroethidium; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HRP, horseradish peroxidase; MTT, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide; MDA, malondialdehyde; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline; NMDA, *N*-methyl-D-aspartate; SOD, superoxide dismutase; ROS, reactive oxygen species; TMP, tetramethylpyrazine.

* Corresponding author. Fax: +86 010 82801434.
E-mail address: shuyanli@bjmu.edu.cn (S.-Y. Li).
[1] Present address: Department of Pathophysiology, Dalian Medical University, Dalian 116027, People's Republic of China.
[2] Present address: Department of Biochemistry, Medical School of Chifeng College, Chi Feng 024001, People's Republic of China.

0891-5849/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2009.12.004

damage in brain and spinal cord by TMP is linked to the scavenging of free radicals [14]. In addition, TMP neuroprotection may involve anti-inflammatory potential [16] and increased transcription of thioredoxin [17]. Therefore, the mechanisms of TMP actions are complicated and uncertain. In this study, we focus on the effect of TMP on mitochondria and demonstrate that TMP administration can alleviate kainate-induced status epilepticus and partially prevent and rescue neuronal loss in the rat hippocampus. The neuroprotective effectiveness of TMP is attributed to the stabilization of mitochondrial function in the cells through scavenging ROS.

Materials and methods

Reagents and materials

Kainate, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline (NBQX), D-2-amino-5-phosphonopentanoic acid (D-AP5), and cyclothiazide (CTZ) were from Tocris Cookson (Bristol, UK). α-Tocopherol (vitamin E) and horseradish peroxidase (HRP) were from Sigma (St. Louis, MO, USA). TMP was from Sigma–Aldrich (Shanghai, China). Fetal bovine serum, Dulbecco's modified Eagle medium (DMEM), Neurobasal medium, and B27 were from Invitrogen (Grand Island, NY, USA). Culture dishes and plates were purchased from Corning Glass (Corning, NY, USA). Other chemicals were obtained from Sigma, unless stated otherwise.

Animal experiments

Animal experimental protocols were approved by the Institutional Committee on Animal Research and were carried out in accordance with the National Institutes of Health guidelines for animal use and care (National Institutes of Health Publication No. 96-23, revised 1996). Male Wistar rats (7 weeks of age, 180 and 200 g) were supplied by the Experimental Animal Center, Peking University Health Science Center, and randomly divided into four groups for the preventive experiments. Rats in the kainate-treated group (KA group) received intraperitoneal (ip) injection of a single dose of kainate (10 mg/kg) in 0.9% NaCl as described [18]. Rats in the control group (saline group) received ip injection of 0.9% (w/v) NaCl of an equal volume. Rats in the TMP and kainate-treated group (TMP + KA group) were pretreated with a single ip injection of TMP (20 mg/kg/day) [17] 12 h before kainate injection, followed by ip injection of the same dose TMP, once a day for 1 week after kainate injection. Rats in the TMP group were injected with TMP in the same way. The severity of seizures was scored for 4 h after kainate injection, according to a previous classification [19]. In the rescue experiments, the rats in the KA + TMP group were treated with TMP (20 mg/kg/day) after 4 h of kainate injection, once a day for 1 week. One week after kainate injection, the animals were anesthetized with pentobarbital sodium (30 mg/kg ip) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and postfixed overnight in the same fixative at 4°C. Coronal brain sections (20 μm thickness) were cut on a freezing microtome and stained with cresyl violet for histological examination of neuronal damage. Nissl-positive undamaged neurons were counted in five coronal brain sections per animal (sections were chosen by unbiased sampling), and the mean number of cells per section was determined such that the value obtained for each rat represents an average total number of neurons counted per section (0.04 mm^2 in the CA1/CA3 regions). Counts were performed by an investigator blinded to treatment status.

Fluoro-JadeB staining

To evaluate neuronal degeneration, Fluoro-JadeB (Chemicon, Temecula, CA, USA) staining was performed as described [20]. Sections were dried on a slide warmer and dipped in solution containing 1% sodium hydroxide in 80% alcohol for 5 min. After a 2-min rinse in 70% (v/v) alcohol and 2-min rinse in dH_2O, sections were transferred to a solution of 0.06% (w/v) $KMnO_4$ for 15 min. After a 2-min rinse in dH_2O, slides were incubated in 0.0004% (w/v) Fluoro-JadeB solution in 0.1% (v/v) acetic acid for 20 min at 4°C. After three washes in dH_2O, sections were air-dried and mounted. Sections were examined with a laser confocal microscope (Leica Microsystems, Germany).

Primary neuronal cultures and kainate exposure

The primary neuronal cultures from hippocampus were prepared as described previously [21,22], with modification. Briefly, hippocampi were removed from newborn Wistar rat pups (postnatal day 1), and dissected hippocampi were digested at 37°C for 15 min in 0.25% trypsin. Hippocampi were then dissociated using fire-polished pipettes and plated at a density of 5×10^5 cells/cm^2 in DMEM with 20% fetal bovine serum onto 96-well or 100-mm dishes precoated with poly-L-lysine and grown in a 37°C incubator with 5% CO_2. After the cells attached to the plates, the culture medium was replaced with Neurobasal medium supplemented with 2% (v/v) B27. Hippocampal neurons were cultured for 12–14 days before kainate (100 μM) exposure. Vitamin E (50 μM) was added twice at 2 (20 μM) and 0.5 h (50 μM) before kainate exposure. TMP was added at 2 (25 μM) and 0.5 h (50 μM) before kainate treatment.

Cell viability assays

Cell viability assays were performed as described [22]. After exposure for the given number of hours, neurons were assayed for viability, using 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma), which was added at a final concentration of 1 mg/ml for 4 h. MTT was removed, and neurons were lysed in 200 μl of dimethyl sulfoxide. Absorbance was measured at 570 nm on a Bio-Rad 680 microplate reader (Hercules, CA, USA). The data are expressed as the percentage of unexposed neurons that remained in the presence of kainate.

Isolation of mitochondria from the rat hippocampus

For analyses of ATP levels and complex I and III activities, isolation of functional mitochondria from the rat hippocampus was performed as described previously [23] with some modifications. Hippocampi were dissected and washed with ice-cold Ca^{2+}-free and Mg^{2+}-free phosphate-buffered saline solution supplemented with 2 mM EGTA. Hippocampi were rapidly immersed in 1 ml of ice-cold mitochondrial isolation buffer containing 210 mM mannitol, 70 mM sucrose, 10 mM Hepes–KOH, pH 7.4, 2 mM EGTA, and 0.1% fatty acid-free bovine serum albumin and then homogenized using a Dounce homogenizer (10 passes with a loose pestle and 10 passes with a tight pestle). After homogenate centrifugation for 10 min at 1060 *g* at 4°C, the supernatant was collected. The pellet was resuspended in the isolation buffer and centrifuged at 1060 *g* for 5 min. The first and second supernatants were pooled together and centrifuged at 14,600 *g* for 10 min at 4°C. The mitochondrial pellet was resuspended in the isolation buffer lacking EGTA and albumin and centrifuged at 14,600 *g* for 10 min. All of the isolation steps were performed on ice. Protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Enzyme assays

The activities of respiratory chain complexes were determined as described previously [24] with modifications using the respective assay kit (Genmed, Shanghai, China). NADH:ubiquinol reductase (complex I) was measured by the decylquinazolinamine-sensitive oxidation of

NADH (340–400 nm, and $\in_{340-400nm} = 5.5\ mM^{-1}\ cm^{-1}$) using decylubiquinone as electron acceptor. The reaction mixture contained 60 μmol $NaPHO_4$, (pH 7.5), 30 μmol NaN_3, 0.15 μmol EDTA, 1.3 mg bovine serum albumin, 0.033 μmol NADH, and 0.063 μmol coenzyme Q in a final volume of 1 ml. Ubiquinol:cytochrome *c* reductase (complex III) was measured by the antimycin-sensitive reduction of cytochrome *c* (550–540 nm, $\in_{550-540nm} = 21.84\ mM^{-1}\ cm^{-1}$). The reaction mixture was the same as in the assay of NADH:ubiquinol reductase. The substrate was 0.063 μmol coenzyme QH, and the acceptor was 0.058 μmol cytochrome *c*.

Determination of glutathione peroxidase (GPX) and glutathione reductase (GR) levels in rat hippocampus and primary hippocampal neurons was performed as described previously [25] using the respective assay kits (bioEol, Nanjing, China). Protein contents of samples were determined with a BCA protein assay kit on aliquots of homogenate before centrifugation. Determination of GR activity was based on the equation A_{340} min/6220 mol/L^{-1} cm^{-1} = mU/ml, where A_{340} is the change in absorbance per minute at 340 nm, and 6220 mol/L^{-1} cm^{-1} is the molar extinction coefficient of NADPH. Determination of GPX activity was achieved by the equation A_{340} min/6220 mol/L^{-1} cm^{-1} = mU/ml, where A_{340} is the change in absorbance per minute at 340 nm, and 6220 mol/L^{-1} cm^{-1} is the molar extinction coefficient of NADPH. Results for both GR and GPX assays were then adjusted to give data as mU/mg protein. One GR unit reduces 1 μmol of oxidized glutathione (GSSG) per minute at 25°C and pH 7.6. One GPX unit consumes 1 μmol NADPH per minute at 25°C and pH 8.0.

Adenosine triphosphate (ATP) level measurements

ATP was determined luminometrically as described previously [26] using an ATP bioluminescence assay kit (bioEol). Following the provided protocol, the ATP amount of mitochondrial samples was determined by a concentration standard curve, and the values of ATP content were normalized according to the protein concentration for each sample (mol ATP/μg protein) and converted to percentage of untreated controls.

Determination of lipid peroxidation

Lipid peroxidation in rat hippocampus and cultured hippocampal neurons was assessed by measuring the concentration of malondialdehyde (MDA) as previously described [27]. Briefly, hippocampus or cell extracts were prepared and incubated with SDS, acetic acid, and thiobarbituric acid at 95°C for 1 h. 1,1,3,3-Tetraethoxypropane was used as a standard. The protein concentration of hippocampus and cell extracts was determined using the BCA protein assay kit, and lipid peroxidation was calculated as nanomoles of MDA per milligram of protein.

ROS measurement in cultured hippocampal neurons

Production of ROS in neurons was determined as described [28] by using dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA). Cultures were incubated in 10 μM DHE at 37°C for 30 min, washed with 1× D-Hanks', and detected with flow cytometry. Hydrogen peroxide (250 μM) was added directly to the bathing medium, and the cells were incubated for 10 min at 37°C as positive controls.

Measurement of mitochondrial transmembrane potential

Mitochondrial transmembrane potential was measured as described previously [29]. Primary hippocampal neurons were incubated for 30 min at 37°C with PBS containing 5 μM rhodamine 123 (R123). After being washed with PBS, cells were trypsinized at room temperature and resuspended in PBS, and the fluorescence was measured by flow cytometry (excitation, 488 nm; emission, 525 nm) using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data are expressed as the mean linear channel number from three to five experiments, with triplicate tubes within one experiment.

Wavelength spectra of TMP and TMP-oxide

Wavelength spectra of TMP and oxidative TMP within 200–700 nm were recorded on a UV/Vis 2800H photometer (UNICO Instrument, Shanghai, China). TMP (0.2 mM) was dissolved in 100 mM phosphate buffer (pH 6.8) and incubated with hydrogen peroxide (3.0, 6.0, 12.0 mM) and HRP at 37°C for 60 min. The buffer used for dilutions and for the blanks was 100 mM sodium phosphate (pH 6.8). Spectra were recorded at room temperature (25°C). In a 1-ml reaction mix, the final concentrations were 100 mM sodium phosphate, 0.2 mM TMP, 3.0–12 mM hydrogen peroxide, and 50 units of HRP.

TMP oxidation by hydrogen peroxide

In the reaction catalyzed by HRP, hydrogen peroxide was reduced (to H_2O) at the expense of TMP as a substance that is oxidized to TMP-*N*,*N*′-dioxide (Fig. 5A). Based on this reaction, the oxidation of TMP by hydrogen peroxide in the presence of HRP was monitored by the decrease in TMP. Briefly, hydrogen peroxide solution ranged from 3 to 15 mM mixed with 0.2 mM TMP and HRP (50 units/ml) dissolved in 100 mM phosphate buffer (pH 6.8). The reaction containing hydrogen peroxide and HRP was used as a blank (adjusted to 0), and the reaction containing TMP and HRP was used as control. The mixture was shaken up vigorously and left at 37°C for 60 min in the dark. The UV absorbance of reactions was measured at 294 nm. The oxidative percentage of TMP was calculated by dividing the absorbance difference between the control (A_C) and the reaction (A_R) by the absorbance of the control, as the in the following equation:

$$\text{TMP oxidation } (\%) = [(A_C - A_R)/A_C] \times 100$$

Data analyses

Multiple comparisons were examined for significant differences using a one-way analysis of variance, followed by individual comparisons with the Tukey posttest. Statistical significance was set at $P<0.05$.

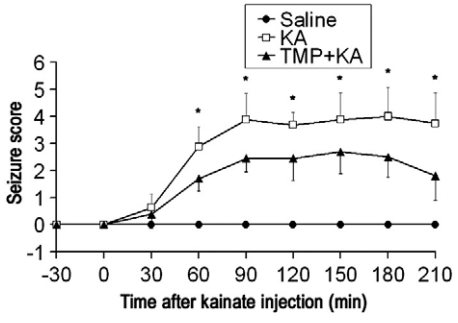

Fig. 1. Alleviation of kainate-induced convulsive behavior in rats by TMP. Male Wistar rats were grouped ($n = 5$ or 6/group) and pretreated (ip injection) with TMP (20 mg/kg) or NaCl (0.9%). Twelve hours after TMP or saline pretreatment, seizures in kainate (KA) and TMP + KA group rats were induced by injection of kainate (10 mg/kg); the rats in the saline group received 0.9% NaCl in the equal volume. The preconvulsive behavior was scored within 4 h after kainate injection. Scores at various time points after seizure induction were analyzed by two independent observers and averaged and statistically compared with one another by two-tailed *t* tests. Data represent means ± SD. *$P<0.05$.

Results

TMP alleviates kainate-induced convulsive behavior in rats

To evaluate protection of TMP against kainate-induced status epilepticus, male Wistar rats (180–200 g) in the TMP + KA group were pretreated with a single ip injection of TMP (20 mg/kg); saline and saline + KA group rats were injected with equal volumes of 0.9% NaCl solution instead of TMP. Twelve hours after TMP or saline pretreatment, seizures were induced by kainate injection (10 mg/kg) or 0.9% NaCl (saline group). The preconvulsive behavior was scored within 4 h after kainate injection. Compared with the saline group rats in which no neurologic anomaly could be observed after saline induction, the preconvulsive behavior in kainate-induced rats occurred initially within 30 min; the seizure scores reached 2.88 at 1 h and substantially increased to 3.7–4 within 3.5 h after kainate injection. Obviously, a delayed onset and decreased score of seizure were observed in the rats receiving TMP pretreatment and kainate

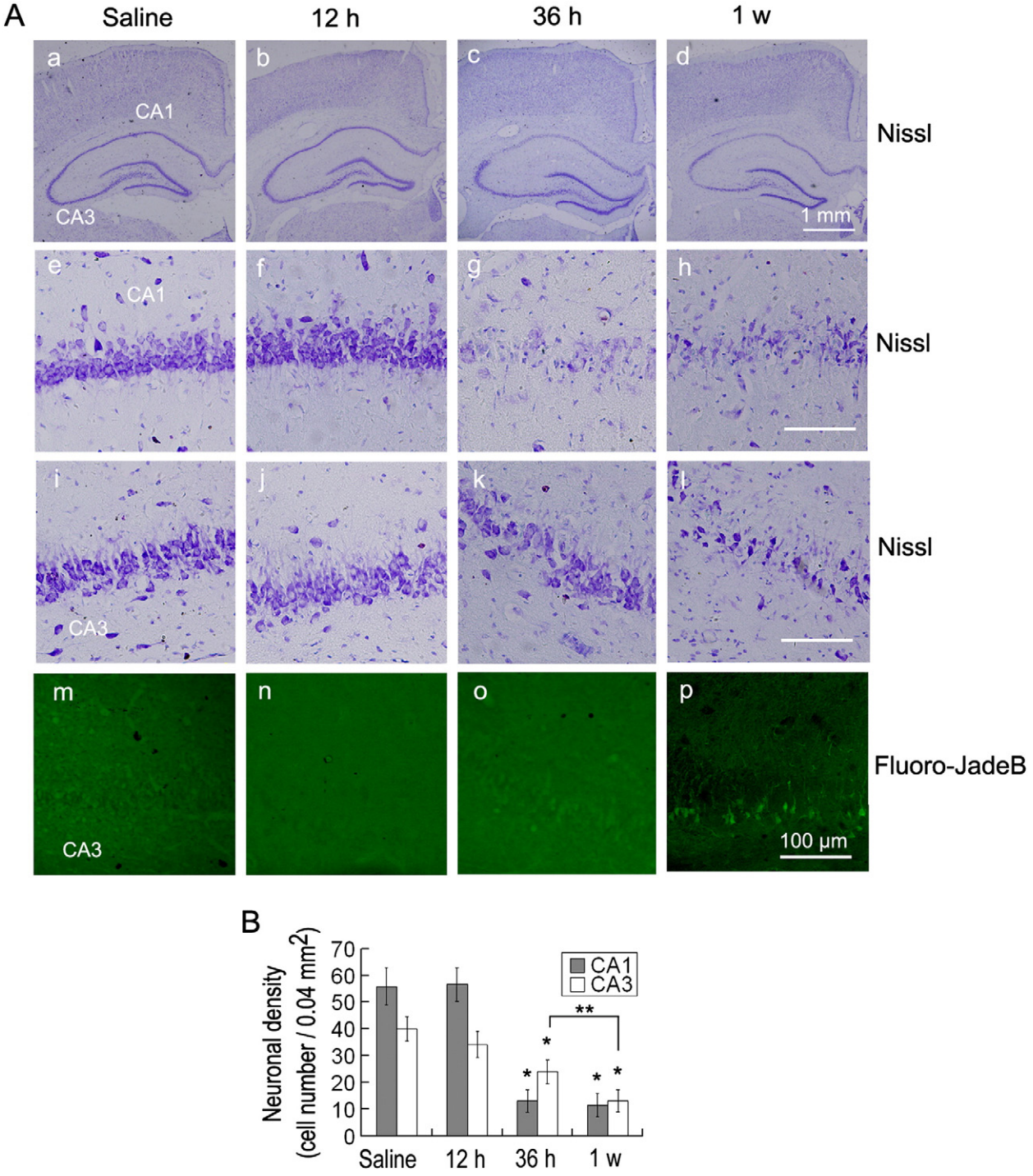

Fig. 2. Kainate-induced neuronal loss in the rat hippocampus. Seizures in male Wistar rats were induced by kainate injection (10 mg/kg, ip). Animals were anesthetized with pentobarbital sodium and transcardially perfused. Brains were removed and postfixed. Coronal brain sections (20-μm thickness) were cut on a freezing microtome and stained with cresyl violet (Nissl) or Fluoro-JadeB. (A) Representative histological examples of cresyl violet- (images a–l) and Fluoro-JadeB- (images m–p) stained brain sections. Saline, saline "induction;" 12 h, 36 h, and 1 w indicate the time after kainate induction. Bar, 1 mm or 100 μm as indicated. (B) Histogram showing neuronal densities in the CA1 and CA3 regions of rat hippocampus measured by Nissl staining and cell counting (cell number/0.04 mm^2). Data represent means ± SD ($n = 5$ or 6). *$P < 0.05$ (kainate vs saline group); **$P < 0.05$ (CA3 36 h vs 1 week).

induction, and the seizure values reached a maximal score of 2.6 at 3 h; thereafter the seizure score dropped to 1.8 (Fig. 1). These results indicate that TMP may alleviate kainate-induced status epilepticus.

TMP prevents kainate-induced neuronal loss in the CA1 and CA3 regions

Kainate-induced status epilepticus leads to delayed selective death of pyramidal neurons in the rat hippocampus [30]. To further evaluate the role of TMP in preventively protecting neurons against kainate excitotoxicity, we first examined the cell lesions in the CA1 and CA3 regions of kainate-injected rats by histochemistry. The cresyl violet staining (Fig. 2A) and the counts of neuron numbers in the two regions (Fig. 2B) showed that kainate caused a marked loss of pyramidal neurons in the CA1 region at 36 h (images c and g in Fig. 2A) and kept a steady decrease by 1 week (images d, h) after kainate-induced seizure. Unlike the CA1 region, neuronal loss in the CA3 region was not so serious at 36 h, and a substantial loss of neurons was detected on day 7 after kainate-induced seizure (images d and l), which was also evidenced by

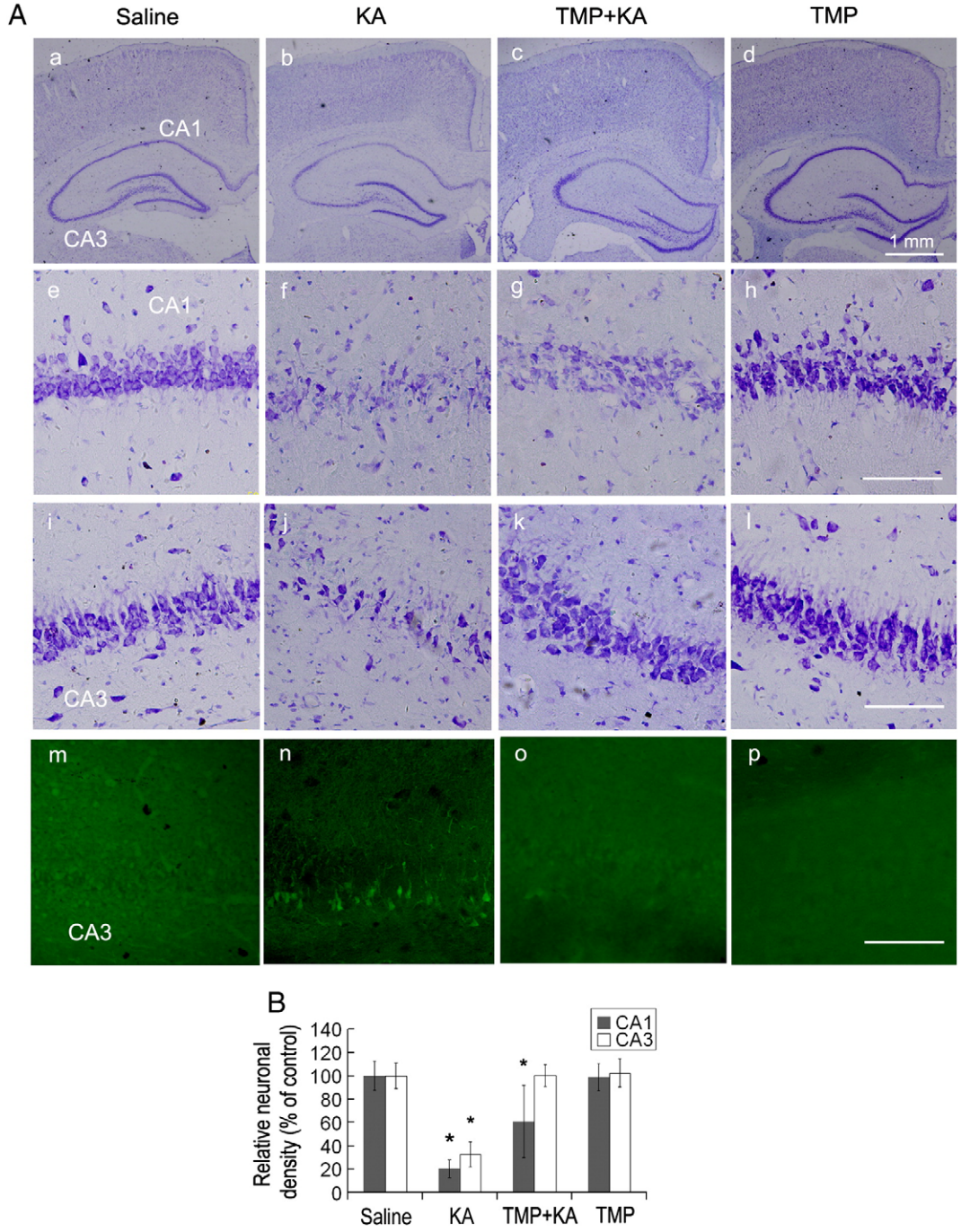

Fig. 3. Partial prevention of kainate-induced neuronal loss in the hippocampus by TMP. Wistar rats were pretreated with TMP (20 mg/kg, ip) or NaCl (0.9%), and seizures were induced by kainate (10 mg/kg, ip) at 12 h after pretreatment, followed by TMP injection (20 mg/kg, ip), once a day for 1 week. Brain sections (20-μm thickness) were prepared and stained as for Fig. 2. (A) Representative histological examples of cresyl violet- (images a–l) or Fluoro-JadeB- (images m–p) stained brain sections. Saline (control), saline pretreatment/saline "induction," $n=5$; KA, saline pretreatment/kainate induction, $n=6$; TMP + KA, TMP pretreatment/kainate induction, $n=6$; TMP, TMP pretreatment/saline "induction," $n=5$. Samples were from rats 1 week after kainate induction. Bar, 1 mm or 100 μm as indicated. (B) Histogram showing relative neuron densities in the CA1 and CA3 regions of the hippocampus measured by Nissl staining and cell counting (cell number/0.04 mm^2). The saline group number is normalized as 1 (100%). Data represent means ± SD. *$P<0.05$ (vs control group).

Fluoro-JadeB staining (images m to p). These results indicate that kainate injection may damage pyramidal neurons in the CA1 and CA3 regions, of which the CA1 region seems to be more vulnerable than CA3.

Next, we compared neuronal loss in the hippocampus among the four groups of rats. The section staining was performed using rats 1 week after induction. The saline (control) and TMP pretreatment did not affect the distribution of neurons in tested areas (images a and d in Figs. 3A and 3B). Compared with the control, kainate treatment led to a loss of the pyramidal neuron numbers in the CA1 (images b and f) and CA3 (images b and j) regions after 1 week of 79.3 and 67.3% of the control, respectively. Notably, TMP pretreatment plus kainate injection resulted in only 39.2 and 0% of kainate-induced neuronal loss in these two regions (images g and k). Similar effects of the reagents on the CA3 region were also observed by Fluoro-JadeB staining (images m to p). These results reveal that TMP pretreatment can partially prevent kainate-induced cell death in the hippocampus.

TMP rescues kainate-induced neuronal loss in the CA3 region

To ascertain whether TMP could rescue kainate-induced neuronal loss in the hippocampus, we administered TMP (20 mg/kg/d, ip) after 4 h of kainate injection, once a day for 1 week, and examined the neuronal loss in the CA1 and CA3 regions. The cresyl violet staining sections (Fig. 4A) and the counts of neuron numbers (Fig. 4B) in rat brain sections showed that kainate treatment led to about 79.3% neuronal loss in the CA1 region and 67.3% loss in CA3 (images b, f, and j in Figs. 4A and 4B), compared with that in the hippocampus of control (saline treated) rat (images a, e, and i). It was noteworthy that administration of TMP after kainate treatment for 4 h rescued about 27.2% of the neuronal loss in the CA3 region, but did not reverse the neuronal loss in the CA1 (images c, g, k). These results indicate that TMP may rescue kainate-induced neuronal loss in the CA3 but not the CA1 region of rat hippocampus.

TMP can function as a reductant to destroy hydrogen peroxide

TMP can be oxidized in vitro to TMP-1,4-(*N*,*N*′)-dioxide in the presence of hydrogen peroxide at high temperature (70°C) [31]. To investigate whether TMP might directly destroy hydrogen peroxide in living cells, we mimicked the intracellular conditions for developing an oxidation–reduction reaction catalyzed by HRP at 37°C for 60 min, in which hydrogen peroxide was reduced at the expense of TMP (Fig. 5A). The spectra of the reactions in the presence of hydrogen peroxide at various concentrations (3–12 mM) were recorded at the wavelengths 200–700 nm. In the reaction containing TMP and HRP, a UV spectrum with double peaks (at 282 and 294 nm) was detected within 250–320 nm (Fig. 5B). When hydrogen peroxide was added, the maximal absorption was markedly decreased in a hydrogen peroxide dose-dependent manner, such that the decrease in UV absorption might be due to oxidation of TMP to TMP-dioxide by hydrogen

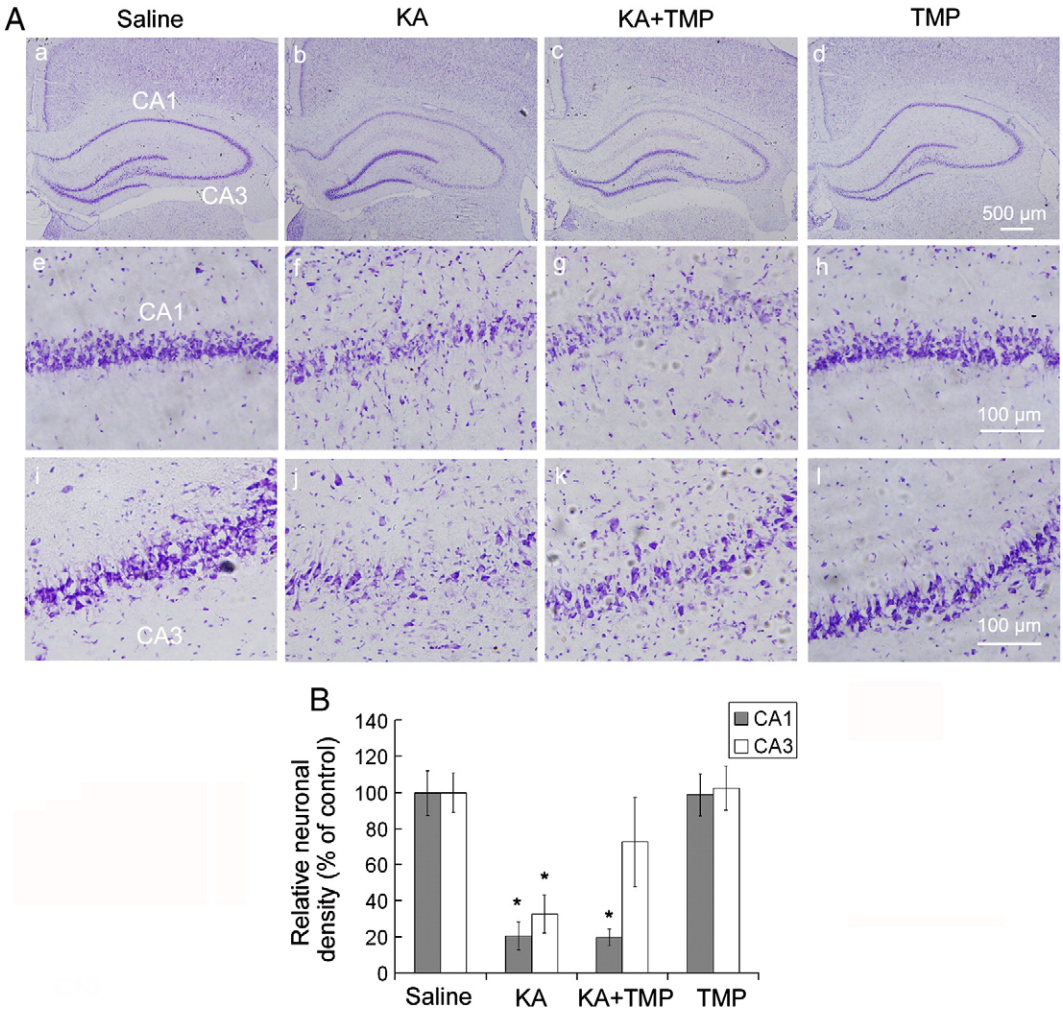

Fig. 4. Rescue of kainate-induced neuronal loss in the CA3 but not the CA1 region by TMP. Seizures in Wistar rats were induced by kainate (10 mg/kg, ip), followed by treatment with or without TMP (20 mg/kg/day, ip), once a day for 1 week. (A) Brain sections were prepared and stained with cresyl violet staining. Bar, 500 or 100 μm as indicated. (B) The neuronal numbers per 0.04 mm^2 in the CA1 and CA3 regions were calculated. Saline (control), saline treatment, $n=5$; KA, kainate induction/saline treatment, $n=6$; KA + TMP, kainate induction/TMP treatment, $n=6$; TMP, TMP treatment, $n=5$. Samples were from rats 1 week after kainate induction. Data represent means ± SD. *$P<0.05$.

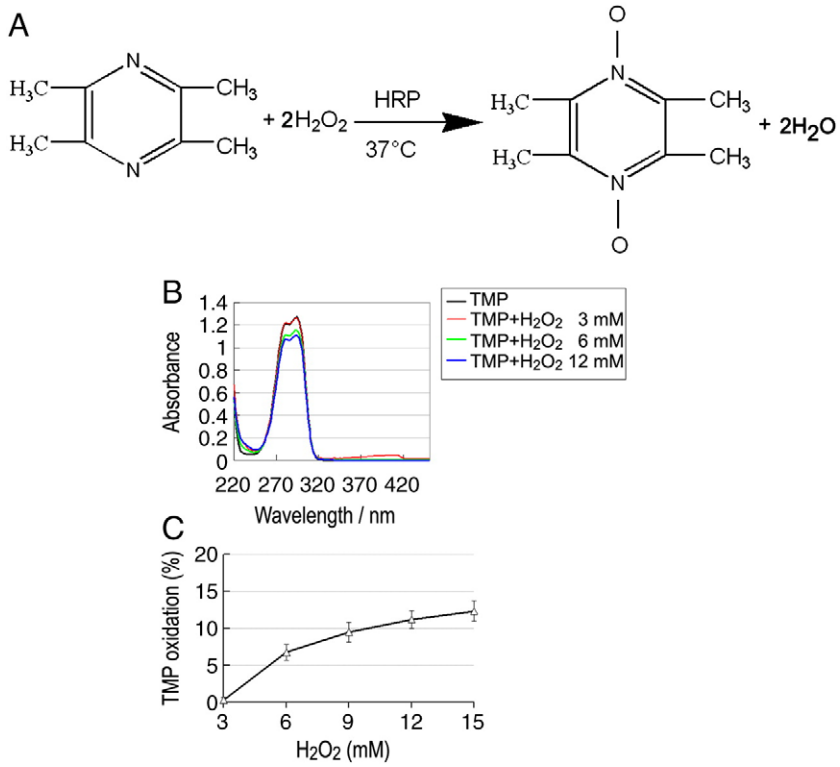

Fig. 5. Direct reduction of hydrogen peroxide by TMP. (A) The oxidation–reduction reaction catalyzed by HRP. The reaction was carried out at 37°C for 60 min, and hydrogen peroxide was reduced at the expense of TMP. (B) The spectra of the reactions in the presence of hydrogen peroxide at various concentrations (3, 6, 12 mM) were recorded at the wavelengths 200–700 nm. (C) Oxidation of TMP by hydrogen peroxide. After the reaction catalyzed by HRP (50 unit), the UV absorption maximum at 294 nm (A_{294}) was determined, and the oxidative rate (%) of TMP was calculated by dividing the absorbance difference between the control (A_C) and the reaction (A_R) by the absorbance of the control. The reaction containing hydrogen peroxide and HRP was used as a blank (0). Data represent means ± SD ($n = 3$).

peroxide in the presence of HRP. This enabled us to use the decrease in TMP in the reactions to detect the capability of TMP to destroy hydrogen peroxide by determining the UV absorption at 294 nm (A_{294}). As shown in Fig. 5C, a hydrogen peroxide concentration-dependent TMP oxidation was observed. These results imply that TMP can function as a reductant to destroy hydrogen peroxide directly.

TMP prevents kainate-induced neuronal death in primary hippocampal neurons

Initially, we examined the effects of TMP on cell viability using primary hippocampal neurons in culture. The MTT method revealed that exposure of hippocampal neurons to 25–100 μM TMP for 24 h had no effect on cell viability (Fig. 6A). Thus, a dose of 50 μM TMP was employed in this study. To demonstrate whether the in vivo protection against kainate-induced neuronal loss was correlated with the role of TMP antioxidation, we examined the effect of TMP on primary cultures of hippocampal neurons. Exposure of hippocampal neurons to 100 μM kainate for 3–24 h elicited a significant decrease in cell survival (Fig. 6B), whereas kainate-induced neuronal loss was prevented by adding 50 and 100 μM TMP, as well as the ROS-quenching agent α-tocopherol (50 μM; Fig. 6C). These results indicate that TMP can protect neurons against kainate-induced cytotoxicity through its antioxidant effect.

TMP reduces ROS accumulation and protects antioxidant enzymes in vitro and in vivo

Excessive activation of glutamate receptors induces oxidative stress in which Ca^{2+}-mediated ROS accumulation plays a critical

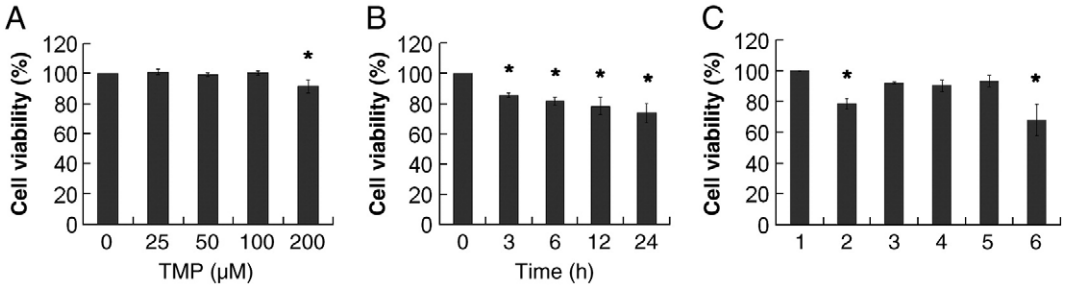

Fig. 6. TMP prevention of kainate-induced neuronal loss in culture. Primary cultured hippocampal neurons were plated at a density of 5×10^5 cells/cm^2 in DMEM with 20% fetal bovine serum onto 96-well dishes and grown in a 37°C incubator with 5% CO_2. Hippocampal neurons were cultured for 12–14 days before experiments (see Materials and methods). (A) Examination of the dose effect of TMP on neuronal viability by MTT assay. Neurons were exposed to TMP at concentrations of 25, 50, 100, or 200 μM for 24 h. (B) Analysis of kainate-induced neuronal loss by MTT assay. Neurons were exposed to 100 μM kainate for 0, 3, 6, 12, or 24 h. (C) Protection of hippocampal neurons against kainate-induced cell loss by TMP. Neurons were exposed to 100 μM kainate (lanes 2–5) with 50 μM vitamin E (lane 3) or 50 (lane 4) or 100 μM (lane 5) TMP. Neurons were exposed to 0.9% NaCl as negative control (lane 1) and to 250 μM H_2O_2 for 10 min as positive control (lane 6). Cell viability at 24 h after kainate exposure was measured by MTT assay. All data represent means ± SD ($n = 5$). *P<0.05.

role in neurotoxicity [1,2]. To explore the mechanisms of TMP neuroprotection, ROS production in kainate-exposed hippocampal neurons was evaluated by DHE staining. DHE is oxidized by intracellular ROS to produce fluorescent ethidium that is subsequently intercalated in DNA, further amplifying its fluorescence [28]. Thus, an increase in DHE oxidation and the subsequent increase in fluorescence are highly suggestive of ROS generation. Flow cytometry analysis of ROS-positive cells (Fig. 7A) showed that, compared with kainate-unexposed neurons (control), the levels of ROS in kainate-exposed neurons were significantly increased ($P<0.05$), as in H_2O_2-exposed neurons (positive control; Fig. 7B). Notably, TMP (50 μM) as well as vitamin E (50 μM) inhibited kainate-induced ROS accumulation markedly. These results indicate that TMP can reduce kainate-induced ROS accumulation in primary hippocampal neurons.

Enzymatic and nonenzymatic antioxidants such as GSH, superoxide dismutase (SOD), GPX, and GR scavenge excessive ROS in the cells. In turn, ROS are able to damage cellular antioxidants. To determine whether ROS accumulation was attributed to suppression of the antioxidant defense system during kainate exposure and whether TMP prevented it, we examined the activities of GPX and GR in cultured hippocampal neurons. Kainate, like hydrogen peroxide, significantly decreased the activities of GPX (10.55 mU/mg; Fig. 7C) and GR (14.22 mU/mg; Fig. 7D) in cultured hippocampal neurons, compared with the normal (basal) levels (21.8 and 23.79 mU/mg in kainate-unexposed neurons). The decrease in both enzyme activities could be reversed by TMP as well as vitamin E. To evaluate the TMP effects in vivo, hippocampi from the four groups of rats (treated as in Fig. 3) were removed 2 days after kainate treatment, and the activities of GPX and GR were measured. Similarly, kainate-suppressed enzyme activities and TMP-preserved enzymes were observed in KA- and KA + TMP-treated rat hippocampus (Figs. 7E and 7F). These results suggest that TMP can protect antioxidant enzyme activities.

TMP alleviates mitochondrial impairment in vitro and in vivo by suppressing lipid peroxidation

ROS induce lipid peroxidation, destroying cellular and subcellular membranes. The level of MDA, a lipid peroxidation end-product, is commonly known as a marker of oxidative stress and the antioxidant status. We thus determined the effects of kainate and TMP on MDA

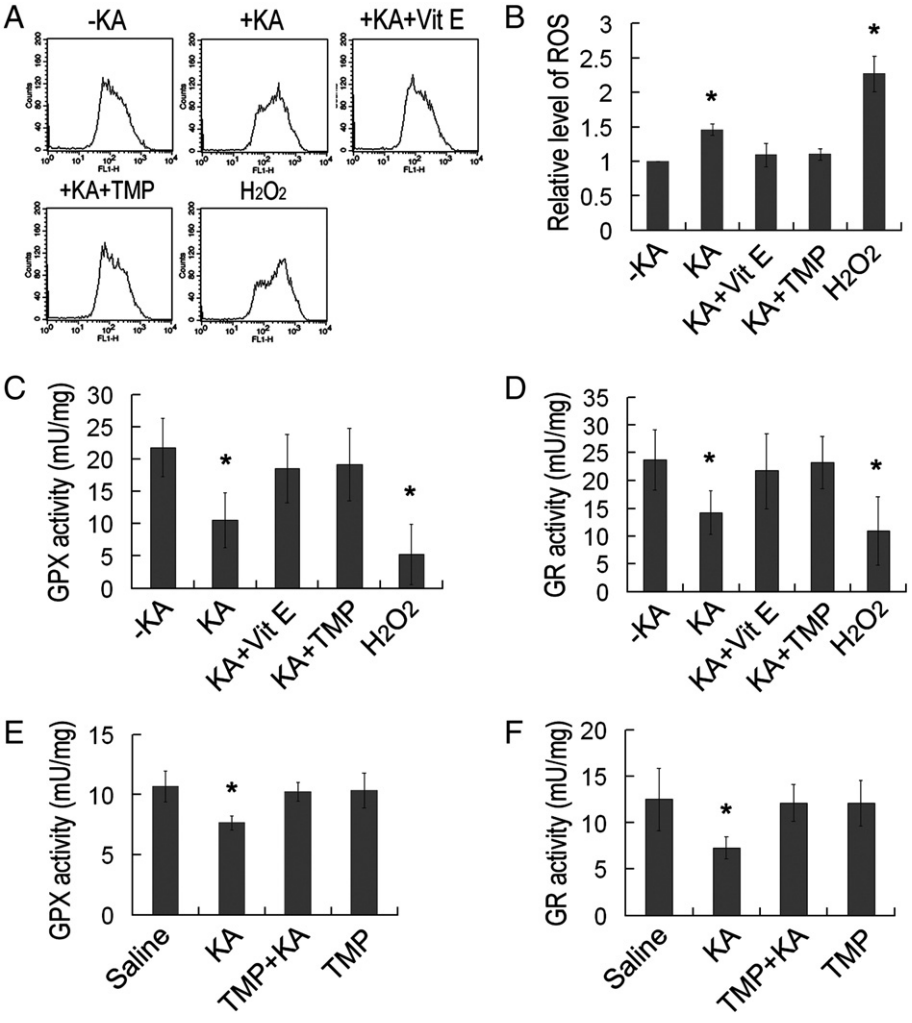

Fig. 7. Prevention of ROS generation and preservation of GPX and GR by TMP. (A) Flow cytometry analysis of ROS generation. Primary hippocampal neurons were cultured as for Fig. 6. Neurons were exposed to 100 μM kainate for 24 h with or without TMP (50 μM) or vitamin E (50 μM) as indicated. Neurons were stained with DHE and analyzed by flow cytometry. Neurons were exposed to 0.9% NaCl as negative control and to 250 μM H_2O_2 for 10 min as positive control. (B) Histogram showing the relative levels of ROS in the experiments show in (A). The ROS levels in control cells were normalized to 1. Data represent means ± SD ($n=3$). *$P<0.05$. (C) Preservation of GPX and (D) GR in hippocampal neurons in culture. Primary hippocampal neurons were treated as in (A) and lysed. After protein determination, enzyme activities were measured using the respective assay kits. Specific activity of GPX is shown as μmol NADPH min^{-1} mg^{-1} protein. Specific activity of GR is shown as μmol GSSG min^{-1} mg^{-1} protein. Data represent means ± SD ($n=3$). *$P<0.05$. (E) Prevention of GPX and (F) GR in the rat hippocampus. Homogenates of rat hippocampi from the animals in the Fig. 3 experiments were prepared and lysed. After protein determination, enzyme activities were measured as in (C) and (D). Data represent means ± SD ($n=3$). *$P<0.05$.

levels in primary hippocampal neurons and the rat hippocampus. Fig. 8A shows that kainate, like hydrogen peroxide, significantly increased the MDA level, to 4.96 nmol/mg on the basal level (without kainate exposure) of 3.51 nmol/mg; Fig. 8B shows that the MDA level in hippocampus from kainate-injected rats was 3.05 nmol/mg, whereas the MDA level in hippocampus from saline (control) group rats was only 2.09 nmol/mg. These results suggest that kainate treatment can induce lipid peroxidation, because of suppressed antioxidant defense and ROS accumulation. Notably, in the same experiments, addition of TMP (50 μM) as well as vitamin E (50 μM) substantially decreased MDA levels to normal levels in primary cultures of hippocampal neurons (Fig. 8A) and rat hippocampus (Fig. 8B), indicating that TMP can inhibit kainate-induced lipid peroxidation in vitro and vivo.

ROS-induced lipid peroxidation may disrupt the structural and functional integrity of neurons. Mitochondria, containing multiple electron carriers capable of producing ROS as well as an extensive network of antioxidant defenses, are both regulators and targets of ROS. Therefore, mitochondrial insults, including oxidative damage itself, can cause an imbalance between ROS production and removal, resulting in net ROS production [32]. We thus quantitatively determined mitochondrial damage in primary hippocampal neurons by flow cytometry. As shown in Figs. 8C and 8D, kainate caused a significant decrease in mitochondrial membrane potential in exposed neurons, which was promoted by CTZ, a desensitization blocker of AMPA receptor, whereas D-AP5, a selective *N*-methyl-D-aspartate (NMDA) receptor antagonist, did not affect it. By contrast, NBQX, a selective and competitive AMPA/KA receptor antagonist, completely blocked the decrease in the membrane potential in exposed neurons. These results indicate that the decrease in mitochondrial membrane potential in exposed neurons is attributable to overactivation of AMPA/KA receptors by kainate. As expected, the antioxidant vitamin E blocked the decrease in the membrane potential. Notably, TMP

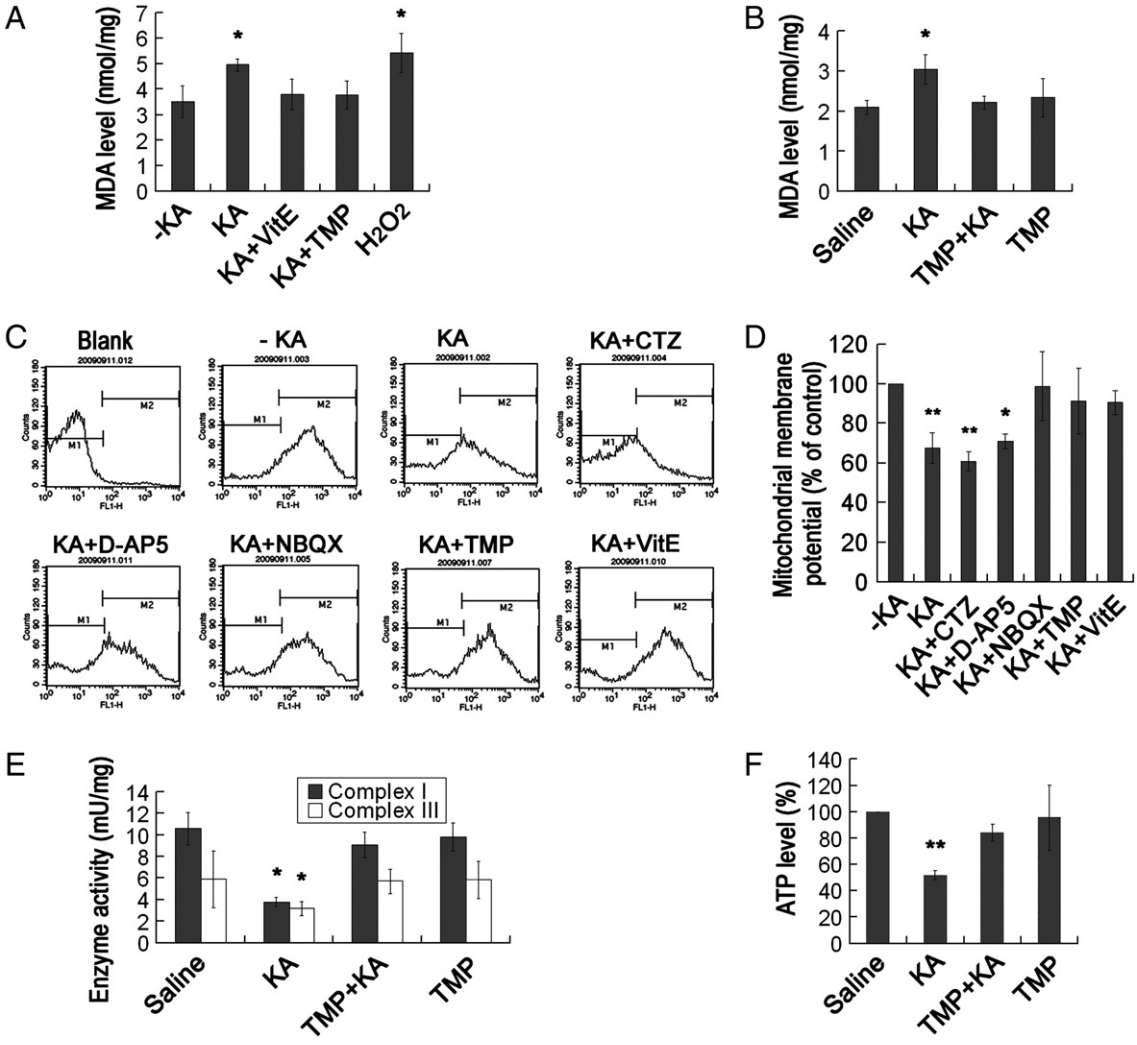

Fig. 8. Prevention of lipid peroxidation and stabilization of mitochondrial functions by TMP. (A) Prevention of lipid peroxidation by TMP in kainate-exposed hippocampal neurons. Primary hippocampal neurons were treated as in Fig. 7A and lysed. MDA levels in cell extracts were determined using 1,1,3,3-tetraethoxypropane as a standard. Lipid peroxidation was calculated as nanomoles of MDA per milligram of protein. Data represent means ± SD ($n = 3$). *$P<0.05$. (B) Prevention of lipid peroxidation by TMP in the rat hippocampus. Homogenates of rat hippocampi from the animals in the Fig. 3 experiments were prepared and lysed 2 days after kainate induction. MDA levels in hippocampal extracts were determined. Data represent means ± SD ($n = 3$). *$P<0.05$. (C) Preservation of mitochondrial membrane potential by TMP. Primary hippocampal neurons were exposed to 100 μM kainate for 24 h with or without TMP (50 μM) or vitamin E (50 μM). To identify AMPA/kainate receptor activation, CTZ (30 μM), D-AP5 (10 μM), and NBQX (5 μM) were used. After R123 staining, mitochondrial membrane potential was analyzed by flow cytometry. (D) Histogram showing the quantitative changes in mitochondrial membrane potential in the experiments shown in (C). Data represent means ± SD ($n = 3$). *$P<0.05$; **$P<0.001$. (E) Preservation of complexes I and III in the rat hippocampus by TMP. Homogenates of rat hippocampi were prepared and lysed as in (B), followed by isolation of mitochondria. After protein determination, the activities of NADH:ubiquinol reductase (complex I) and ubiquinol:cytochrome *c* reductase (complex III) were measured by the respective kits. Specific activities of complexes I and III were adjusted to give data as mU/mg protein. Data represent means ± SD ($n = 5$). *$P<0.05$. (F) Protection of ATP production in the rat hippocampus by TMP. Isolation of mitochondria of rat hippocampi was the same as for (E). After protein determination, the levels of ATP were quantitatively determined by a bioluminescence assay kit and calculated from a concentration standard curve, normalized as mol ATP/μg protein and converted to percentage of untreated controls. Data represent means ± SD ($n = 4$). **$P<0.001$.

(50 μM) also abolished the kainate-reduced mitochondrial membrane potential, indicating that TMP can protect neurons against kainate-induced mitochondrial impairment.

To further demonstrate that ROS-induced lipid peroxidation disrupted the metabolic integrity of neurons and TMP protects hippocampal neurons against kainate-induced mitochondrial dysfunction, mitochondria were isolated from the hippocampus of the four groups of rats (Fig. 3), followed by analyzing the activities of respiratory chain complexes I and III from mitochondrial membranes. The activity of NADH:ubiquinol reductase (complex I) was measured by the decylquinazolinamine-sensitive oxidation of NADH, and ubiquinol:cytochrome *c* reductase (complex III) was measured by the antimycin-sensitive reduction of cytochrome *c*. As shown in Fig. 8E, compared with the complexes I (10.61 mU/mg) and III (5.91 mU/mg) activities in the saline group (control) rats, the enzymatic activities of both complexes in kainate-treated rat hippocampus were reduced to 3.79 and 3.19 mU/mg, respectively, whereas no decreases in the complex activities could be observed in TMP- and kainate + TMP-treated rat hippocampus. Supporting these results, kainate-treated rats showed a decrease in the generation of ATP by 48%, whereas TMP and kainate + TMP group rats showed a reduced generation of ATP that was only about 4.4 and 15.87% that of saline group rats, respectively (Fig. 8F). The results from Fig. 8 suggest that TMP can protect neurons against kainate-induced mitochondrial dysfunction.

Discussion

In this study we demonstrate that TMP can partially alleviate kainate-induced status epilepticus in rats and prevent and rescue pyramidal neuron loss in the hippocampus. The prevention and rescue of partial neuronal loss in the hippocampus by TMP are attributed to the preservation of mitochondrial function. The stabilization of mitochondrial function by TMP is linked to the function of TMP as a reductant/antioxidant to quench ROS. These results indicate that stabilization of mitochondrial function by TMP can protect against kainate-induced oxidative lesions in the rat hippocampus. Our data support the notion that mitochondrial dysfunction and oxidative stress occur early in neurodegenerative diseases, and this dysfunction has a causal role in disease pathogenesis [3]. In addition, our data suggest that TMP and its derivatives may have the potential to be developed as a therapeutic drugs for neurodegenerative diseases.

Multiple roles for TMP have been reported. In a model of ischemia brain injury in animals, TMP neuroprotection is correlated with increased transcription of thioredoxin [17] and anti-inflammatory potential [16]. In spinal cord ischemia/reperfusion injury, TMP reduces apoptosis by regulating Bcl-2 and Bax expression [14]. During rat subarachnoid hemorrhage, TMP inhibits the activation of the caspase-dependent apoptosis pathway [33]. TMP blocks H_2O_2-induced apoptosis of PC12 cells by regulating Bcl-2 family members, suppressing cytochrome *c* release and caspase activation [34]. Anti-apoptosis by *Ligusticum chuanxiong* (the origin of TMP) is mediated by activating the PKA/CREB-dependent pathway in serum deprivation-induced PC12 death [35]. Early reports suggest that TMP can increase cerebral blood flow [36] and improve the blood viscosity [15] and microcirculation [37] of experimental animals. Although a variety of mechanisms in various models have been suggested, we believe that the core of TMP neuroprotection against kainate-induced oxidative lesions is linked to the preservation of mitochondrial function.

Mitochondria are critical regulators of cell death, a key feature of neurodegenerative diseases [3]. The inner mitochondrial membrane harbors the four major respiratory complexes (I–IV) and the F_0F_1-ATP synthase (complex V). Complexes I (NADH:ubiquinone oxidoreductase), III (ubiquinol:cytochrome *c* oxidoreductase), and IV (cytochrome *c* oxidase) transduce the energy of nutritional compounds,

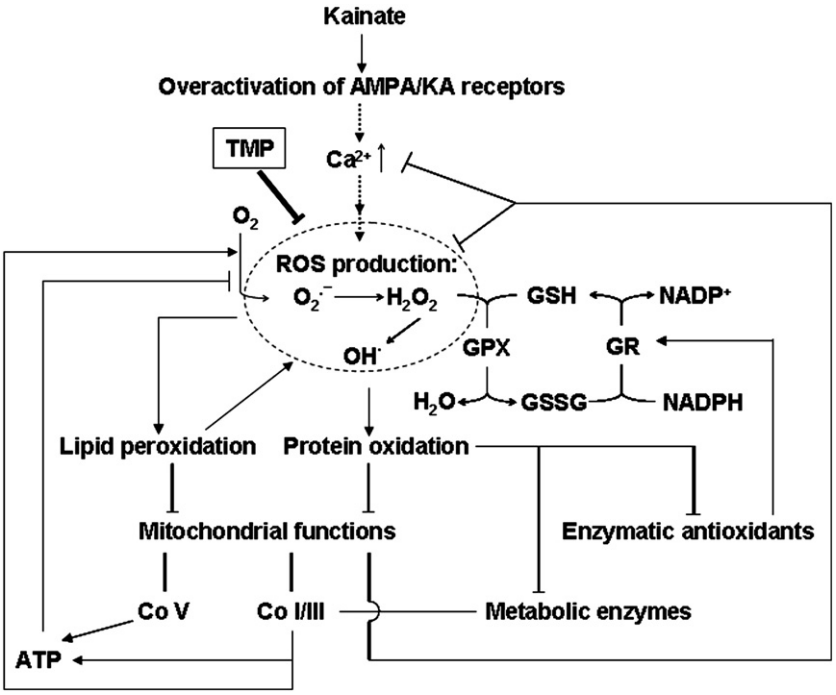

Fig. 9. Schematic presentation of proposed signaling pathways in TMP protection of hippocampal neurons against kainate-induced oxidative stress. Overactivation of AMPA/KA receptors induces mitochondrial and cellular Ca^{2+} overload, leading to the production of ROS, which ultimately causes oxidative stress [1,2]. Increased ROS cause lipid peroxidation and protein (and DNA) damage and disrupt the structural and functional integrity of mitochondria (mitochondrial dysfunction), which is associated with cell death. Production of free radicals by lipid peroxidation and damage of enzymatic antioxidants such as GPX and GR by protein oxidation further aggravate ROS accumulation. An imbalance between ROS formation and ATP production due to the suppression of complexes I, III, and V can also increase ROS accumulation. Because mitochondria are critical regulators [3], mitochondrial dysfunction fails to regulate Ca^{2+} homeostasis and ROS metabolism, further aggravating oxidative stress. TMP prevents ROS production by scavenging $O_2^{\cdot-}$, H_2O_2, and hydroxyl radicals ($OH^{\cdot}$), thereby elevating internal antioxidant GSH defenses, maintaining ATP production, and enhancing cell survival. ↑, promotion; ⊤, suppression.

leading to ATP generation. Normally, the electron flow in mitochondria may produce ROS. On the other hand, mitochondria contain an extensive antioxidant defense system to detoxify the ROS, which depends mainly on GSH, SOD, and GPX [32]. GPX can convert hydrogen peroxide into water and oxygen in the presence of GSH, and GR catalyzes the reduction of GSSG to GSH (Fig. 9). Oxidative stress is an imbalance between ROS production and the antioxidant defense system. The pro-oxidant–antioxidant imbalance in kainate-induced oxidative stress may be due to Ca^{2+} increases in the cytosol and the mitochondria. Elevated intraneuronal Ca^{2+} activates peptidases such as calpain I, which can catalyze the enzymatic conversion of xanthine dehydrogenase to xanthine oxidase; the metabolism of purine bases by xanthine oxidase yields $O_2^{\cdot-}$ and H_2O_2. This reaction may become quite prominent, because kainate receptor agonists cause a depletion of ATP [2]. Like ROS generation, antioxidant defenses are tied to the redox and energetic state of the mitochondrion. We did find the decrease in ATP generation to be coupled with the decrease in complex I and III activities (Figs. 7E and 7F) and GPX and GR activities (Figs. 6C–6F) in kainate-treated hippocampal neurons and the rat hippocampus, suggesting mitochondrial dysfunction during kainate-induced neurotoxicity. Decreased activities of GPX and GR result in ROS accumulation during AMPA/kainate receptor activation. Increased ROS can damage lipids, proteins, and DNA and disrupt membrane integrity [6,7], leading to cellular and mitochondrial dysfunctions that are associated with cell death [3]. Lipid peroxidation produces toxic aldehyde products and free radicals, which further aggravate oxidative stress and degenerative processes. Antioxidants can protect cells from excitotoxicity by scavenging ROS and increasing intracellular cysteine levels [38]. We found that TMP could directly function as a reductant (Fig. 8) to quench ROS and reduce ROS accumulation, thereby protecting antioxidant enzymes (Figs. 6C–6F), preventing lipid peroxidation (Figs. 7A and 7B), and preserving the structural and functional integrity of mitochondria (Figs. 7C–7F). Subsequently, maintaining mitochondrial function by TMP may regulate cellular Ca^{2+} homeostasis and attenuate Ca^{2+}-mediated ROS generation, thereby protecting biomolecules, including membrane lipids, essential cellular proteins, and DNA. In these ways, TMP improves cellular redox status, protecting from neuronal death in kainate-induced oxidative stress (Fig. 9). Non-NMDA-receptor-mediated neurotoxicity is more complex [2]. In addition, superoxide activates inducible nitric oxide synthase, leading to the production of nitric oxide and the formation of peroxynitrites, which ultimately causes oxidative/nitrosative stress [2,39]. Furthermore, ROS originating in mitochondria are thought to be a major source of endogenous nuclear DNA damage [40]. Therefore, further study of the detailed signaling pathways may provide a better understanding of TMP protection against kainate-induced oxidative stress.

Although the basicity of pyrazine is weak, TMP (2,3,5,6-tetramethylpyrazine) has an increased basicity, because of the repelling of electrons by the four methyl groups (Fig. 5A). Therefore, the two nitrogen atoms with an unshared electron pair can function as an electron donor (Lewis base). TMP can be oxidized in vitro to TMP-1,4-(*N*,*N*′)-dioxide in the presence of hydrogen peroxide and at 70°C [31]. This, however, is not the case in living cells. To mimic the intracellular environment, we developed a reduction–oxidation reaction catalyzed by HRP (Fig. 5A). The reduction state of TMP exhibited a UV-absorption maximum at 294 nm (A_{294}), while the A_{294} of the oxidation state was decreased (Fig. 5B), which can be used to determine the reducing capability of TMP. In the presence of HRP, TMP could directly be oxidized at 37°C in a hydrogen peroxide dose-dependent manner (Fig. 5C), indicating that TMP is a hydrogen peroxide scavenger. This can explain how TMP decreases ROS accumulation, protecting against oxidative damage in the rat hippocampus.

Systemic administration of kainate in rodents increases ROS production and induces cell death by both necrotic and apoptotic pathways. The CA1 and CA3 regions and the hilus of the dentate gyrus are particularly sensitive to the excitotoxicity of kainate [41–43]. We found that the loss of pyramidal neurons in the CA1 region was greater than that in the CA3 (Figs. 2–4). Consistent with this, protection of CA3 neurons by TMP was more effective than that of CA1 (Figs. 3 and 4), indicating that CA1 neurons are more sensitive to kainate-induced excitotoxicity. Probably, there are several reasons for the different sensitivities of CA1 and CA3 neurons. First, the different sensitivities of the two regions might be associated with the cell death pathways. For instance, p53 promotes cell death via multiple pathway-apoptotic cell death in CA1 neurons and necrotic cell death in CA3 in kainate-treated mice [44]. Second, our observation that TMP rescued kainate-induced neuronal loss in the CA3 but not CA1 region suggests earlier occurrence of irreversible injury in CA1 neurons. The irreversible injury to CA1 might involve specific changes in gene expression. This inference is supported by a previous finding that GluR2 antisense combined sublethal ischemia produces virtually complete loss of CA1 pyramidal cells and partial loss of CA3 pyramidal cells [45]. Third, CA3 and CA1 regions have distinct anatomical and physiological features. The CA3 region has dense excitatory recurrent synaptic connections between pyramidal cells; CA1 pyramidal cells receive a large number of excitatory synaptic inputs from CA3 pyramidal cells through Schaffer collaterals. This may allow CA1 cells to be sequence-sensitive [46]. The precise explanation for the different injuries to CA1 and CA3 during kainate-induced excitotoxicity remains to be found.

Acknowledgments

We thank Miss Yang Lei (Department of Anatomy, Peking University Health Science Center, China) for her assistance in brain section management. This work was supported in part by National Natural Science Foundation of PR China grants (30771225, 30671062, 30971449) and the Education Committee of Beijing Teaching Reinforcing Plan.

References

[1] Choi, D. W. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1:**623–634; 1988.
[2] Coyle, J. T.; Puttfarcken, P. Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262:**689–695; 1993.
[3] Lin, M. T.; Beal, M. F. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* **443:**787–795; 2006.
[4] Vila, M.; Przedborski, S. Targeting programmed cell death in neurodegenerative diseases. *Nat. Rev. Neurosci.* **4:**365–375; 2003.
[5] Tanaka, H.; Grooms, S. Y.; Bennett, M. V.; Zukin, R. S. The AMPAR subunit GluR2: still front and center-stage. *Brain Res.* **886:**190–207; 2000.
[6] Choi, D. W. Calcium and excitotoxic neuronal injury. *Ann. N. Y. Acad. Sci.* **747:** 162–171; 1994.
[7] Siesjö, B. K.; Bengtsson, F.; Grampp, W.; Theander, S. Calcium, excitotoxins, and neuronal death in the brain. *Ann. N. Y. Acad. Sci.* **568:**234–251; 1989.
[8] Herrlich, P.; Bohmer, F. D. Redox regulation of signal transduction in mammalian cells. *Biochem. Pharmacol.* **59:**35–41; 2000.
[9] Hall, A. G. The role of glutathione in the regulation of apoptosis. *Eur. J. Clin. Invest.* **29:**238–245; 1999.
[10] Lu, C. S.; Yin, M. H.; Huang, Y. H.; Pao, C.; Sung, C. F. Radix salviae miltiorrhizae and Rhizoma ligustici wallichii in coronary heart disease. *Chin. Med. J. (English)* **4:** 43–46; 1978.
[11] Guo, S. K.; Chen, K. J.; Qian, Z. H.; Weng, W. L.; Qian, M. Y. Tetramethylpyrazine in the treatment of cardiovascular and cerebrovascular diseases. *Planta Med.* **47:**89; 1983.
[12] Jiao, F.; Cong, K.; Li, Y. Observation on ischemic cerebrovascular disease (113 cases) treated by injection of ligustrazine. *J. Pract. Tradit. Chin. Med.* **20:**174–175; 2004.
[13] Zhu, X. Q.; Liu, G. H.; Hu, J. X. The anti-epileptic effect of tetramethylpyrazine and its relationship to interleukin-1 receptor. *Lishizhen Med. Materia Med. Res.* **18:** 2927–2928; 2007.
[14] Fan, L. H.; Wang, K. Z.; Cheng, B.; Wang, C. S.; Dang, X. Q. Anti-apoptotic and neuroprotective effects of tetramethylpyrazine following spinal cord ischemia in rabbits. *BMC Neurosci.* **7:**48; 2006.
[15] Chang, F. C.; Huang, Y. T.; Lin, H. C.; Hong, C. Y.; Lin, J. G.; Chen, K. J. Beneficial effects of combined terlipressin and tetramethylpyrazine administration on portal hypertensive rats. *Can. J. Physiol. Pharmacol.* **77:**618–624; 1999.

[16] Liao, S. L.; Kao, T. K.; Chen, W. Y.; Lin, Y. S.; Chen, S. Y.; Raung, S. L.; Wu, C. W.; Lu, H. C.; Chen, C. J. Tetramethylpyrazine reduces ischemic brain injury in rats. *Neurosci. Lett.* **372:**40–45; 2004.
[17] Zhu, X. L.; Xiong, L. Z.; Wang, Q.; Liu, Z. G.; Ma, X.; Zhu, Z. H.; Hu, S.; Gong, G.; Chen, S. Y. Therapeutic time window and mechanism of tetramethylpyrazine on transient focal cerebral ischemia/reperfusion injury in rats. *Neurosci. Lett.* **449:** 24–27; 2009.
[18] Savaskan, N. E.; Bräuer, A. U.; Kühbacher, M.; Eyüpoglu, I. Y.; Kyriakopoulos, A.; Ninnemann, O.; Behne, D.; Nitsch, R. Selenium deficiency increases susceptibility to glutamate-induced excitotoxicity. *FASEB J.* **17:**112–124; 2003.
[19] Zhang, X.; Gelowitz, D. L.; Lai, C. T.; Boulton, A. A.; Yu, P. H. Gradation of kainic acid-induced rat limbic seizures and expression of hippocampal heat shock protein-70. *Eur. J. Neurosci.* **9:**760–769; 1997.
[20] Schmued, L. C.; Albertson, C.; Slikker, W. J. Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. *Brain Res.* **751:**37–46; 1997.
[21] Lissin, D. V.; Carroll, R. C.; Nicoll, R. A.; Malenka, R. C.; von Zastrow, M. Rapid, activation-induced redistribution of ionotropic glutamate receptors in cultured hippocampal neurons. *J. Neurosci.* **19:**1263–1272; 1999.
[22] Li, S. Y.; Ni, J. H.; Xu, D. S.; Jia, H. T. Down-regulation of GluR2 is associated with Ca^{2+}-dependent protease activities in kainate-induced apoptotic cell death in cultured rat hippocampal neurons. *Neurosci. Lett.* **352:**105–108; 2003.
[23] Kushnareva, Y. E.; Wiley, S. E.; Ward, M. W.; Andreyev, A. Y.; Murphy, A. N. Excitotoxic injury to mitochondria isolated from cultured neurons. *J. Biol. Chem.* **280:**28894–28902; 2005.
[24] Tzagoloff, A.; Akai, A.; Needleman, R. B.; Zulch, G. Assembly of the mitochondrial membrane system: cytoplasmic mutants of Saccharomyces cerevisiae with lesions in enzymes of the respiratory chain and in the mitochondrial ATPase. *J. Biol. Chem.* **250:**8236–8242; 1975.
[25] White, A. R.; Collins, S. J.; Maher, F.; Jobling, M. F.; Stewart, L. R.; Thyer, J. M.; Beyreuther, K.; Masters, C. L.; Cappai, R. Prion protein-deficient neurons reveal lower glutathione reductase activity and increased susceptibility to hydrogen peroxide toxicity. *Am. J. Pathol.* **155:**1723–1730; 1999.
[26] Kumar, V.; Bal, A.; Gill, K. D. Impairment of mitochondrial energy metabolism in different regions of rat brain following chronic exposure to aluminium. *Brain Res.* **1232:**94–103; 2008.
[27] White, A. R.; Multhaup, G.; Maher, F.; Bellingham, S.; Camakaris, J.; Zheng, H.; Bush, A. I.; Beyreuther, K.; Masters, C. L.; Cappai, R. The Alzheimer's disease amyloid precursor protein modulates copper-induced toxicity and oxidative stress in primary neuronal cultures. *J. Neurosci.* **19:**9170–9179; 1999.
[28] Gross, E. R.; LaDisa Jr., J. F.; Weihrauch, D.; Olson, L. E.; Kress, T. T.; Hettrick, D. A.; Pagel, P. S.; Warltier, D. C.; Kersten, J. R. Reactive oxygen species modulate coronary wall shear stress and endothelial function during hyperglycemia. *Am. J. Physiol. Heart Circ. Physiol.* **284:**H1552–1559; 2003.
[29] Poppe, M.; Reimertz, C.; Düssmann, H.; Krohn, A. J.; Luetjens, C. M.; Böckelmann, D.; Nieminen, A. L.; Kögel, D.; Prehn, J. H. Dissipation of potassium and proton gradients inhibits mitochondrial hyperpolarization and cytochrome c release during neural apoptosis. *J. Neurosci.* **21:**4551–4563; 2001.
[30] Grooms, S. Y.; Opitz, T.; Bennett, M. V.; Zukin, R. S. Status epilepticus decreases glutamate receptor 2 mRNA and protein expression in hippocampal pyramidal cells before neuronal death. *Proc. Natl. Acad. Sci. USA* **97:**3631–3636; 2000.
[31] Cheng, X. C.; Liu, X. Y.; Xu, W. F.; Guo, X. L.; Ou, Y. Design, synthesis, and biological activities of novel Ligustrazine derivatives. *Bioorg. Med. Chem.* **15:**3315–3320; 2007.
[32] Andreyev, A. Y.; Kushnareva, Y. E.; Starkov, A. A. Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Moscow)* **70:**200–214; 2005.
[33] Gao, C.; Liu, X.; Liu, W.; Shi, H.; Zhao, Z.; Chen, H.; Zhao, S. Anti-apoptotic and neuroprotective effects of tetramethylpyrazine following subarachnoid hemorrhage in rats. *Auton. Neurosci.* **141:**22–30; 2008.
[34] Cheng, X. R.; Zhang, L.; Hu, J. J.; Sun, L.; Du, G. H. Neuroprotective effects of tetramethylpyrazine on hydrogen peroxide-induced apoptosis in PC12 cells. *Cell Biol. Int.* **31:**438–443; 2007.
[35] Lin, Y. L.; Lee, Y. C.; Huang, C. L.; Lai, W. L.; Lin, Y. R.; Huang, N. K. Ligusticum chuanxiong prevents rat pheochromocytoma cells from serum deprivation-induced apoptosis through a protein kinase A-dependent pathway. *J. Ethnopharmacol.* **109:**428–434; 2007.
[36] Feng, M. G.; Feng, G. H.; Zhou, Q. G. Effects of methylhesperidin on coronary, renal and cerebral circulation in dogs [in Chinese]. *Acta Pharmacol.* **9:**548–550; 1988.
[37] Dai, X. Z.; Bache, R. J. Coronary and systemic haemodynamic effect of tetramethylpyrazine in the dog. *J. Cardiovasc. Pharmacol.* **7:**841–849; 1985.
[38] Carriedo, S. G.; Sensi, S. L.; Yin, H. Z.; Weiss, J. H. AMPA exposures induce mitochondrial Ca^{2+} overload and ROS generation in spinal motor neurons in vitro. *J. Neurosci.* **20:**240–250; 2000.
[39] Ansari, M. A.; Keller, J. N.; Scheff, S. W. Protective effect of Pycnogenol in human neuroblastoma SH-SY5Y cells following acrolein-induced cytotoxicity. *Free Radic. Biol. Med.* **45:**1510–1519; 2008.
[40] Huang, M. E.; Kolodner, R. D. A biological network in Saccharomyces cerevisiae prevents the deleterious effects of endogenous oxidative DNA damage. *Mol. Cell* **17:**709–720; 2005.
[41] Ben-Ari, Y.; Cossart, R. Kainate, a double agent that generates seizures: two decades of progress. *Trends Neurosci.* **23:**580–587; 2000.
[42] Choi, D. W. Excitotoxic cell death. *J. Neurobiol.* **23:**1261–1276; 1992.
[43] Frerking, M.; Nicoll, R. A. Synaptic kainate receptors. *Curr. Opin. Neurobiol.* **10:** 342–351; 2000.
[44] Morrison, R. S.; Wenzel, H. J.; Kinoshita, Y.; Robbins, C. A.; Donehower, L. A.; Schwartzkroin, P. A. Loss of the p53 tumor suppressor gene protects neurons from kainate-induced cell death. *J. Neurosci.* **16:**1337–1345; 1996.
[45] Oguro, K.; Oguro, N.; Kojima, T.; Grooms, S. Y.; Calderone, A.; Zheng, X.; Bennett, M. V.; Zukin, R. S. Knockdown of AMPA receptor GluR2 expression causes delayed neurodegeneration and increases damage by sublethal ischemia in hippocampal CA1 and CA3 neurons. *J. Neurosci.* **19:**9218–9227; 1999.
[46] Yoshida, M.; Hayashi, H. Emergence of sequence sensitivity in a hippocampal CA3–CA1 model. *Neural Netw* **20:**653–667; 2007.