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Research report

Neuroprotection of co-activation of GABA receptors by preventing caspase-3 denitrosylation in KA-induced seizures

Xue-Wen Wei[a], Hui Yan[a], Bo Xu[a], Yong-Ping Wu[b], Chong Li[a,*], Guang-Yi Zhang[a,*]

[a] Jiangsu Key Laboratory of Brain Disease Bioinformation and Research Center of Biochemistry and Molecular Biochemistry, Xuzhou Medical College, Xuzhou 221002, China
[b] Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical College, Xuzhou 221002, China

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ABSTRACT

Previous studies have demonstrated that kainic acid (KA)-induced seizures can cause the enhancement of excitation and lead to neuronal death in rat hippocampus. Co-activation of the inhibitory GABA receptors can attenuate the excitatory JNK3 apoptotic signaling pathway via inhibiting the increased assembly of the GluR6-PSD-95-MLK3 signaling module induced by KA in epileptic rat hippocampal CA1 and CA3 regions. Caspase-3 is a cysteine protease located in both the cytoplasm and mitochondrial intermembrane space that is a central effector of many apoptotic pathways. We designed experiments to elucidate the underlying molecular mechanisms of procaspase-3 activation and neuroprotection of co-activation of GABA receptors against neuronal death induced by KA. In this study, we show that co-activation of GABA receptors can attenuate the Fas/FasL apoptotic signaling pathway and inhibit the increased of thioredoxin reductase activity induced by KA, subsequently inhibit the activation of procaspase-3 by diminishing the denitrosylation of its active-site thiol and decreasing the cleavage of the caspase-3 zymogen to its active subunits. These results indicate that co-activation of GABA receptors results in neuroprotection by preventing caspase-3 denitrosylation in KA-induced seizure of rats.

1. Introduction

Epilepsy is one of the most common neurological disorders that affect people of all ages worldwide [6]. Prolonged epileptic seizure or status epilepticus results in subcellular changes that lead to neuronal damage and death in the hippocampus [8,16]. One model of excitotoxic cell death is the administration of the glutamate mimetic, kainic acid (KA) [25].

KA is a potent exogenous agonist of kainate receptors and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors. The effect of KA administration on the central nervous system has been widely studied, especially in the hippocampal region, using both in vivo and in vitro models, and it has been reported that the CA1 and CA3 regions and the hilus of the dentate gyrus are particularly sensitive to the excitotoxicity of KA [3,9,15]. KA-induced seizures in rodents have been widely used as a model of human temporal lobe epilepsy on the basis of both behavioral and pathological similarities [2].

Kainate receptors are composed of five subunits, glutamate receptor 5 (GluR5), GluR6, GluR7, KA1, and KA2 [25]. Previous studies have indicated that the RLPGKETMA motif of the C terminus of GluR6 can bind to the PDZ1 domain of the postsynaptic density protein PSD95/SAP90 through specific interaction [25]. Studies have also shown that MLK3, an upstream kinase of JNK [25], can interact with the SH3 (Src homology) domain of PSD95 and GluR6, PSD95, and MLK3 form a signaling module and facilitate MLK3 and JNK phosphorylation and activation in vitro [25]. This signaling module exists in epileptic rat hippocampal CA1and CA3 regions and KA-induced neuronal death is mediated by the GluR6-PSD95-MLK3 signaling module [27].

GABA is the major inhibitory neurotransmitter in the brain, where it activates both ionotropic and metabotropic transduction mechanisms. The former are mediated primarily through the activation of GABAA receptors, whereas the metabotropic activities of GABA are produced via GABAB receptors [18]. Co-application of muscimol (GABAA receptor agonist) and baclofen (GABAB receptor agonist) can inhibit the assembly of the GluR6-PSD95-MLK3 signaling module in KA-induced seizures [26].

The Fas/Fas-ligand (FasL) pathway is the best-studied receptor-mediated death signaling pathway. Stimulation of the receptor

Abbreviations: KA, kainic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; GABA, γ-Aminobutyric acid; FasL, Fas-ligand; FADD, Fas-associated death domain; DISC, death-inducing signaling complex; Trx, thioredoxin; TrxR, Trx reductase; SNO, S-nitrosothiol; NOS, nitric oxide synthase; JNK, c-Jun N-terminal kinase; MLK3, mixed lineage kinase 3; DMSO, dimethyl sulfoxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

* Corresponding authors at: Research Center for Biochemistry and Molecular Biology, Xuzhou Medical College, No. 84 West Huai-hai Road, Xuzhou 221002, China. Tel.: +86 516 8574 8486; fax: +86 516 8574 8486.
E-mail addresses: lichong@xzmc.edu.cn (C. Li), gyzhang@xzmc.edu.cn (G.-Y. Zhang).

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Fas by its natural ligand, FasL, or Fas activating antibody results in recruitment of two key signaling proteins, the adapter protein FADD (Fas-associated death domain, also called MORT-1) and the initiator cysteine protease caspase-8, which together form the death-inducing signaling complex (DISC). Proteolytic autoactivation of DISC results in activation of the effecter caspases, including the key effecter caspase, caspase-3. Activated caspase-3 cleaves DNA repair enzymes, cellular and nuclear structural proteins, endonucleases, and many other cellular constituents, culminating in effective cell death [31,38,41].

Caspases play distinct roles in the apoptotic cascade and as many as 14 different caspases have been described so far [7,11,12,24,30]. They are expressed as inactive enzymes, and activated during apoptosis [39]. Caspase-3 has been shown to inactivate proteins that protect living cells from programmed cell death and is a central player of many apoptotic pathways, as it is required for DNA fragmentation and morphological changes associated with apoptosis such as cellular shrinkage and blebbing [13,22]. It is inhibited by S-nitrosylation on a cysteine residue in its active site. Stimulation of the cell surface "death" receptor Fas causes the denitrosylation of this subpopulation of caspases, leading to apoptosis [29].

Our previous studies have demonstrated that KA enhanced the assembly of the GluR6-PSD-95-MLK3 module; increased the autophosphorylation of MLK3 and the phosphorylation of MKK7, JNK3, c-Jun, and Bcl-2; raised the expression of FasL; and caused the release of Bax from Bcl-2/Bax dimmers and the release of cytochrome c from mitochondria. Consequently, the activation of caspase-3 led to delayed neuronal death in the hippocampal CA1/CA3 subfield [33,35,40]. Co-activation of the inhibitory GABA receptors could attenuate the excitatory JNK3 apoptotic signaling pathway via inhibiting the increased assembly of the GluR6-PSD-95-MLK3 signaling module induced by KA [26]. Therefore, we proposed to identify whether stimulation of the inhibitory GABA receptors has neuroprotective effect by preventing caspase-3 denitrosylation and further elucidate the underlying molecular mechanisms of procaspase-3 activation and TrxR regulation.

2. Materials and methods

2.1. Antibody and reagents

The following primary antibodies from Santa Cruz Biotechnology, Inc. were used: rabbit polyclonal anti-FasL (sc-6237), rabbit polyclonal Fas (M-20) (sc-716), and rabbit polyclonal anti-actin(sc-10731). Rabbit polyclonal anti-caspase-3 antibody (#9662) and cleaved caspase-3 (Asp175) antibody (#9661) were obtained from Cell Signaling Biotechnology, Inc. Baclofen (B5399) was bought from Sigma. The secondary antibodies used in our experiment were goat anti-mouse IgG and goat anti-rabbit IgG, which were also purchased from Sigma.

2.2. Seizure model

Adult male Sprague-Dawley rats weighing 200–300 g were used. The experimental procedures were approved by the local legislation for the ethics of experiments on animals. Seizures were induced by intracerebroventricular injection of KA (0.6 μg/10 μl) dissolved in sterile saline. Animals were monitored behaviorally for seizures after injection. The seizures were scored using a modified scale devised by Racine [37]: (1) behavioral arrest and staring spells, (2) head bobbing and gnawing, (3) unilateral forelimb clonus, (4) bilateral forelimb clonus, (5) severe seizures with loss of postural control, and (6) seizure-induced death [37]. Only animals with stage 4 or 5 seizure were used for this study. Controls were injected with sterile saline.

2.3. Administration of drugs

KA was injected through intracerebroventricular infusion (anteroposterior, 0.8 mm; lateral, 1.5 mm; and depth, 3.5 mm from the bregma) and saline was used as control. Muscimol (1 mg/kg, i.p.) [10] and baclofen (20 mg/kg, i.p.) [20] was administrated to the rats 40 min before KA injection, as well as saline control. The animals were divided into four groups: animals in 'Saline' group were administrated with saline both through intracerebroventricular infusion and intraperitoneally injection; in 'KA' group only KA was administrated; in 'saline + KA' group, saline was administrated through i.p. and KA was injected through intracerebroventricular infusion; in 'M + B + KA' group, muscimol and baclofen was administrated through i.p. and KA was injected through intracerebroventricular infusion.

2.4. Sample preparation

Rats were decapitated with an overdose of anesthetic (chloral hydrate) immediately at 3 h and 6 h after KA injection. The timepoints selected were based on that in KA induced seizure model, caaspase-3 was cleaved and activated, and reached its peak activity at 6 h timepoint after KA injection [27], while peak of caspase-3 S-nitrosylation was found at 3 h timepoint (data in submission). The hippocampi were dissected from both hemispheres separately then separated into CA1 and CA3/dentate gyrus (DG) regions by cutting along the hippocampal fissure and quickly frozen in liquid nitrogen. The hippocampi were homogenized in 1:10 (w/v) ice-cold homogenization buffer containing 50 mM MOPS (pH 7.4; Sigma), 100 mM KCl, 320 mM sucrose, 50 mM NaF, 0.5 mM $MgCl_2$, 0.2 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 (Sigma), 20 mM sodium pyrophosphate, 20 mM β-phosphoglycerol, 1 mM p-nitrophenyl phosphate, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotin, and 5 μg/ml pepstatin A. The homogenates were centrifuged at 800 × *g* for 10 min at 4 °C. Supernatants were collected, and protein concentration was determined by the method of Lowry et al. [28]. Samples were stored at −80 °C and were thawed only once until used.

2.5. Immunoprecipitation

Tissue homogenates (400 μg of protein) were diluted 4-fold with immunoprecipitation buffer (50 mM HEPES buffer (pH 7.4) containing 10% glycerol, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4). Samples were pre-incubated for 1 h with 20 μl of protein A-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) at 4 °C and centrifuged to remove proteins that had adhered nonspecifically to protein A. The supernatants were incubated with 1–2 μg of primary antibodies for 4 h or overnight at 4 °C. Protein A was added to the tube for another 2-h incubation. Samples were centrifuged at 10,000 × *g* for 2 min at 4 °C, and the pellets were washed three times with immunoprecipitation buffer. Bound proteins were eluted by boiling at 100 °C for 5 min in SDS-PAGE loading buffer and then isolated by centrifugation. The supernatants were used for immunoblot analysis.

2.6. Immunoblotting

Proteins were separated on polyacrylamide gels and then electrotransferred to nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). After blocking for 3 h in Tris-buffered saline with 0.1% Tween 20 (TBST) and 3% bovine serum albumin, membranes were incubated overnight at 4 °C with primary antibodies in TBST containing 3% bovine serum albumin. Membranes were then washed and incubated with alkaline phosphatase-conjugated secondary antibodies in TBST for 2 h and developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color substrate (Promega Corp.).The density of the bands on the membrane was scanned and analyzed with LabWorks image analysis software (UVP, Inc.).

2.7. S-nitrosylation assay

S-nitrosylation was detected using the Biotin-Switch method as previously described by Jaffrey et al. [21] with minor modifications. Briefly, tissue was homogenized in HEN buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% Nonidet P-40, 150 mM NaCl, 1 mM PMSF, protease inhibitor mixture) then mixed with an equal volume of methyl methanethiosulfonate (MMTS) buffer (25 mM HEPES, pH 7.7, 0.1 mM EDTA, 10 μM neocuproine, 5% SDS, 20 mM MMTS) and incubated at 50 °C for 20 min with frequent vortexing. After the free MMTS is removed by cold acetone precipitation, the precipitates are resuspended in HENS buffer (25 mM HEPES, pH7.7, 0.1 mM EDTA, 10 μM neocuproine, 1% SDS). After the addition of 2 volumes of neutralization buffer (20 mM HEPES, pH 7.7, 1 mM EDTA, 100 mM NaCl, 0.5% Triton X-100), the samples are then modified with biotin in the following buffer (25 mM HEPES, pH 7.7, 0.1 mM EDTA, 1% SDS, 10 μM neocuproine, 10 mM ascorbic acid sodium salt, and 0.2 mM biotin-HPDP). After free biotin-HPDP was removed by cold acetone precipitation, biotinylated proteins are absorbed to streptavidin–agarose. The streptavidin absorbates are then eluted by β-mercaptoethanol (100 mM), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with an anti-caspase-3 antibody.

2.8. Histological analysis

Rats were deeply anesthetized with chloral hydrate (350 mg/kg, intraperitoneally) and then were perfused transcardially with ice-cold normal saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) after 7 days of KA injection. Brains were removed quickly and further fixed with the same fixation solution overnight at 4 °C. Post-fixed brains were embedded in paraffin, followed by preparation of coronal sections (5 μm thick) using a microtome. The paraffin-embedded brain sections were deparaffinized with xylene and rehydrated