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Protocol

Electron spin resonance assay of ascorbyl radical generation in mouse hippocampal slices during and after kainate-induced seizures

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Abstract

As an index of oxidative status, we analyzed ascorbyl radical generation during and after kainate-induced seizures in mouse hippocampus, using an ESR spectrometer equipped with a special tissue-type quartz cell. A specific doublet ESR spectrum was observed after seizures, and the *g* value and the hyperfine coupling constant (hfcc) of the spectrum were identical with those of ascorbyl radical itself. Antiepileptic zonisamide inhibited the generation of ascorbyl radical accompanying the seizures.

Theme: Disorders of the nervous system
Topic: Epilepsy: basic mechanisms

Keywords: Ascorbyl radical; Kainate-induced seizure; Superoxide anion radical; Hippocampus; Zonisamide

1. Type of research

+ Isolation of hippocampus from brain and preparation of hippocampal slices
+ Induction of seizures by intraperitoneal injection of kainate
+ Generation of superoxide anion radical in the hippocampus
+ Testing the effect of zonisamide, an antiepileptic drug
+ ESR spectrometry with spin-trapping reagent.

Systemic or intracerebral administration of kainate (KA), a glutamate receptor agonist, produces severe and stereotype behavioral convulsions and brain damage syndrome, and rodents injected with KA are used as a model for human temporal lobe epilepsy and status epilepticus [1,2,11]. The excessive stimulation of glutamate receptors accelerates Ca^{2+} influx [4] and may lead to calcium events, e.g., activation of nitric oxide synthase (NOS) and phospholipase A_2, which generate free radicals such as nitric oxide (NO) and superoxide anion radical ($O_2^{\bullet -}$). Reactive oxygen species (ROS) are known to play a role in KA-induced neuronal damage. The hydroxyl radical was demonstrated in cultured rat retinal neurons exposed to KA by electron spin resonance (ESR) spin-trapping method [5]. Ample evidence suggests that KA could generate ROS in vivo such as increased levels of superoxide dismutase (SOD) [8,9], malonaldehyde [15], protein oxidation [3], lipid radicals [12], and iron ion [14] in the brain after KA treatment. Ascorbate is an antioxidant in a hydrophilic phase and is used for protection against oxidative stress. After one electron oxidation, ascorbate becomes an ascorbyl radical. The latter can be observed in circulating blood of rats using ESR spectrometry [13]. In this study, ascorbyl radical was measured in mouse hippocampal

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slices after KA-induced seizures using a special ESR technique.

2. Time required

2.1. Preparation of hippocampal slices

(1) Intraperitoneal administration of ascorbate or zonisamide solution at 30 min before KA injection, respectively.
(2) KA intraperitoneal injection.
(3) Appearance of seizures 15–30 min after KA administration.
(4) Sacrifice of the mice during the first tonic–clonic convulsions (during seizure) by decapitation. Mice were also sacrificed if no strong seizures appeared within 30 min of KA administration.
(5) Careful separation of the whole brain from the occipital region.
(6) Separation and placement of hippocampal slices on the glass and cleaning the tissue surface with ice-cold buffer/saline.

2.2. Tissue handling and spectrometry

(1) Dry the surface of the tissue-type quartz cell and clean the cover glass.
(2) Place the tissue on the tissue-type quartz cell: 5–10 min.
(3) Additions of spin-trapping reagent or another solution before setting the cover glass with greasy edge: several tens of microliters.
(4) Dry the surface of the tissue-type quartz cell and check for leaks.
(5) Carefully insert the tissue-type quartz cell into the ESR cavity.
(6) Readjust spectrometer after insertion: several tens of tens of seconds.

ESR spectra measuring time: 2–8 min at room temperature.

3. Materials

3.1. Animals

The study was conducted in 6- to 8-week-old male ddY mice weighing 20–25 g. They were purchased from an animal breeder (Saitama Jikkenn Dobutsu, Saitama, Japan) and housed for 1 week before experimentation in standard animal facilities with free access to food and water. Food was withdrawn 24 h before experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Sojo University.

3.2. Chemicals

Methylcellulose 1500 cP (cP: centipoises) and ascorbic acid (ascorbate) were purchased from Wako Chemicals (Osaka, Japan). Dimethyl sulfoxide (DMSO) was from Nacalai Tesque Inc. (Kyoto, Japan). 6-Hydroxypurine (hypoxanthine) and KA (kainate: 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine) were from Sigma (St. Louis, MO). Xanthine oxidase (20 U/ml: from cow milk) was from Roche Diagnostics (Mannheim, Germany), 5, 5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was from Labotec (Tokyo, Japan), 1, 2-benzisoxazole-3-methanesulfonamide (zonisamide) was from Dainippon Pharmaceutical Co. (Osaka). Superoxide dismutase (SOD: 2650 ± 500 U/mg: from bovine blood) was from Funakoshi (Tokyo).

3.3. Solutions

All chemicals were of analytical quality. Methylcellulose (MC, 0.25%) and saline were used as solvents. KA was dissolved in MC (0.25%) just before the administration and was injected intraperitoneally (i.p.) at a dose of 53.3 mg/kg body weight. Zonisamide and ascorbate were also dissolved in MC (0.25%). The rinse solution for brain tissue was ice-cold 0.1 M phosphate buffer/saline, pH 7.8. SOD solution was 200 U/ml with 0.1 M phosphate buffer/saline, pH 7.8.

3.4. Equipment

+ Electron spin resonance (ESR) spectrometer (JEOL, JES-FA200 spectrometer, Tokyo). The ESR spectrometry conditions used to estimate the $O_2^{\bullet -}$ and ascorbyl radical with spin-trapping reagent were as follows: microwave frequency = 9385.501–9392.20 MHz, microwave power = 4.00 mW, field center 334.297–335.32 mT, sweep width ±5.00 mT, modulation frequency width = 0.0800 mT/100.00 kHz, sweep time = 4.0–8.0 min, amplitude 500–1500, and time constant = 0.3–1.0 s, at room temperature.
+ ESR universal cavity (JEOL, ES-UCX2:TE_{011} mode cavity) with X-band microwave units (8.750–9.650 GHz).
+ ESR standard marker with manganese oxide (MnO) powder (JEOL DATUM, MO7-FB-4).
+ Aqueous sample cell (JEOL, ES-LC12); effective sample volume: up to 150 μl.
+ A tissue-type quartz cell (Labotec, Tokyo); length 40 mm, width 5.0 mm, depth 0.5 mm (Fig. 1).
+ Isotropic spectrum simulation in ESR spectrometer system (JEOL, JES FA series).

4. Detailed procedure

4.1. Drug administration

Tonic–clonic convulsions appeared 15–30 min after KA (53.3 mg/kg) injection, and mice were sacrificed by