Neuroscience 193 (2011) 300–309

PROTECTIVE EFFECT OF 2,2′-DITHIENYL DISELENIDE ON KAINIC ACID-INDUCED NEUROTOXICITY IN RAT HIPPOCAMPUS

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Abstract—In this study, we investigated the effects of 2,2′-dithienyl diselenide (DTDS), an organoselenium compound, against seizures induced by kainic acid (KA) in rats. Rats were pretreated with DTDS (50 or 100 mg/kg) by oral route 1 h before KA injection (10 mg/kg, intraperitoneal). Our results showed that DTDS (100 mg/kg) was effective in increasing latency for the onset of the first clonic seizure episode induced by KA, as well as in decreasing the appearance of seizures and the Racine's score. DTDS also caused a decrease in the excitatory electroencephalographic (EEG) changes, resulting from KA exposure in hippocampus and cerebral cortex of rats. Besides, elevated reactive species (RS) and carbonyl protein levels and Na^+, K^+-ATPase activity in hippocampus of rats treated with KA were ameliorated by DTDS (50 and 100 mg/kg). Lastly, as evidenced by Cresyl-Violet stain, DTDS (100 mg/kg) elicited a protective effect against KA-induced neurodegeneration in rat hippocampus 7 days after KA injection. In conclusion, the present study showed that DTDS attenuated KA-induced status epilepticus in rats and the subsequent hippocampal damage. **© 2011 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: rats, kainic acid, seizures, hippocampus, oxidative stress, selenium.

Epilepsies are among the most frequent CNS disorders affecting approximately 1.5% of the population worldwide (Majores et al., 2004). Temporal lobe epilepsy (TLE) is the most common form of adult epilepsy that involves the limbic structures (Hargus et al., 2011). Because of similar basic features, experimental models have allowed the determination of the basic molecular and cellular mechanisms of epileptogenesis and its relation to brain damage. This is particularly exemplified in the field of TLE (Ben-Ari and Cossart, 2000).

Systemic administration of kainic acid (KA) in rodents induces an epilepsy syndrome similar to human TLE, with mesial temporal sclerosis, spontaneous seizures, as well as significant deficits in learning and memory (Ben-Ari, 1985). Ionotropic glutamate receptor agonists, such as KA, typically increase cell death. Stimulation of glutamate receptors triggers sodium (Na^+) influx and neuronal depolarization, which lead to an opening of voltage-dependent calcium (Ca^{2+}) channels and stimulation of Ca^{2+} influx (Park et al., 2004). Elevated Ca^{2+} concentration generates reactive oxygen species (ROS), inducing injury and neuronal death (Allison and Pratt, 2003).

Oxidative stress is emerging as a key factor that not only results from seizures but may also contribute to epileptogenesis. In this sense, oxidative stress may be a brand new way for the development of new drugs that are neuroprotective. Synthetic antioxidants that protect mitochondrial targets and decrease neuronal death may be useful supplements for the clinical management of patients with status epilepticus (SE) or intractable epilepsy (Waldbaum and Patel, 2010). This is relevant because TLE is among the most frequent types of drug-resistant epilepsy (Engel, 2001).

The essential trace element selenium is of fundamental importance to human health. As a constituent of the small group of selenocysteine-containing selenoproteins, selenium elicits important structural and enzymatic functions (Papp et al., 2007). The pharmacotherapeutic efficacy of selenium has been confirmed in a number of experimental models of brain diseases (Yousuf et al., 2007; Ishrat et al., 2009). It is noteworthy that in a selenium-deficient diet, the brain exhibits a high priority to conserve this element, indicating the importance of this trace element to normal brain function (Schweizer et al., 2004a,b). With regard to epilepsy, an association between selenium deficiency and epilepsy has been reported (Ashrafi et al., 2007a,b). In fact, oral selenium supplementation has improved the clinical state and electroencephalogram of children with intractable epileptic seizures and low blood selenium concentrations (Weber et al., 1991; Ramaekers; et al., 1994). Reinforcing this idea, Savaskan et al. (2003) reported that selenium is effective in protecting neurons from primary damage in the course of excitotoxic lesions induced by KA in rats, suggesting that an adequate sele-

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ANOVA, analysis of variance; DCHF, fluorescent dichlorofluorescein; DCHF-DA, 2′, 7′-dichlorofluorescein diacetate; DNPH, dinitrophenylhydrazine; DTDS, 2,2′-dithienyl diselenide; EEG, electroencephalographic; GABA, gamma-aminobutyric acid; GPx, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; KA, kainic acid; NMDA, N-methyl-D-aspartate; NPSH, nonprotein sulfhydryl; Pi, inorganic phosphate; p.o., orally; ROS, reactive oxygen species; RS, reactive species; SE, status epilepticus; S1, low-speed supernatant; TLE, temporal lobe epilepsy.

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doi:10.1016/j.neuroscience.2011.07.038

nium supplementation is an important strategy to prevent the detrimental effects of excitotoxicity. Therefore, we believe that compounds containing selenium in their structure could also eventually reduce excitotoxic insults, such as seizures.

The interest in organoselenium chemistry and biochemistry has increased, mainly because these compounds show important biological activities, such as neuroprotection (Nogueira et al., 2004; Nogueira and Rocha, 2010). 2,2′-Dithienyl diselenide (DTDS), an organoselenium compound, has shown to be a promising antioxidant in rat brain homogenate *in vitro* (CF. Bortolatto, unpublished observations). Based on the above considerations, the objective of the present study was to investigate the protective effect of DTDS, an organoselenium compound, against seizures induced by KA in rats.

EXPERIMENTAL PROCEDURES

Animals

Adult male Wistar rats (200–300 g) were obtained from a local breeding colony. Animals were housed in cages with free access to food and water. They were kept in a separate animal room on a 12-h light/12-h dark cycle, with lights on at 7:00 AM, in an air-conditioned room (22±2 °C). Animal care and all experimental procedures were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 80–23, revised in 1996) and in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources from the Federal University of Santa Maria, Brazil. All efforts were made to minimize the number of animals used and their suffering.

Chemicals

The compound DTDS (Fig. 1) was prepared according to the literature method (Tiecco et al., 2000). Analysis of hydrogen-1 nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra showed that the obtained compound presented spectroscopic data in full agreement with its assigned structure. The chemical purity of this compound (99.9%) was determined by GC-MS. KA, phenobarbital, chloral hydrate, 2', 7'-dichlorofluorescein diacetate (DCHF-DA), dinitrophenylhydrazine (DNPH), 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB), β-nicotinamide adenine dinucleotide phosphate reduced (NADPH) tetrasodium salt, ouabain, adenosine triphosphate (ATP), bovine serum albumin were obtained from Sigma (St. Louis, MO, USA). All other chemicals were obtained from analytical grade and standard commercial suppliers. The DTDS chemical characteristics are given below:

Yield: 0.023 g (40%). H^1 NMR ($CDCl_3$, 200 MHz), δ (ppm): 7.50–7.47 (dd, J=5.3 Hz, 2H), 7.23–7.24 (dd, J=3.5 Hz, 2H), 7.00 (d, J=8.8 Hz, 1H), 7.00 (d, J=1.6 Hz, 2H). C^{13} NMR ($CDCl_3$, 50 MHz), δ (ppm): 136.91, 132.91, 128.09, 125.518. MS (EI, 70 eV) m/z (relative intensity): 325 (34), 162 (100), 160 (53), 119 (24), 93 (4), 84 (27), 70 (32), 51 (5).

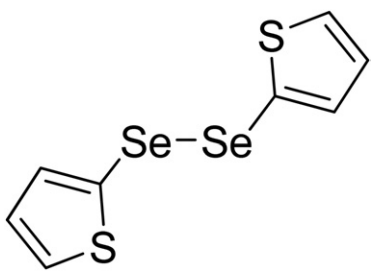

Fig. 1. Chemical structure of DTDS.

Drug treatment and evaluation of behavioral changes

In a section of experiments, the animals were randomly assigned to six groups (n=8–10 per group). The rats were orally (p.o.) treated by gavage with DTDS (50 or 100 mg/kg dissolved in canola oil) or canola oil, and after 1 h the animals received i.p. KA (10 mg/kg dissolved in saline) or saline. The drugs were administered at a fixed volume of 1 ml/kg of body weight. The protocol used to elicit KA-induced seizures was based on previous studies (Morales-Garcia et al., 2009; Shin et al., 2009).

After injection of KA, the animals were put in individual cages, and they were attended for 4 h to evaluate the appearance of seizures, latency for the onset of the first seizure episode, as well as convulsive behavior according to the following rating scale described by Racine (1972): 0=no reaction; 1=stereotype mouthing, eye blinking and/or mild facial clonus; 2=head nodding and/or severe facial clonus; 3=myoclonic jerks in the forelimbs; 4=clonic convulsions in the forelimbs with rearing; and 5=generalized clonic convulsions associated with loss of balance, which were expressed as Racine's score. Latency for the onset of the first seizure episode was assumed as fourth stage of Racine's score. Because the dose of 100 mg/kg of DTDS showed the most effective protection against seizure induced by KA, this dose was chosen for subsequent electroencephalographic and histological analysis.

Electroencephalographic (EEG) surgical procedures

A subset of animals (n=4 per group) was surgically implanted with electrodes under stereotaxic guidance. Animals were then anesthetized with Equitesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3 ml/kg, i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, two screw electrodes were placed bilaterally over the parietal cortex along with a ground lead positioned over the nasal sinus (coordinates from bregma: AP −4.5 mm; L 2.5 mm) (Paxinos and Watson, 1986). Bipolar nichrome wire Teflon-insulated depth electrodes (100 μm in diameter) were implanted 1 mm above the CA1 region of the dorsal hippocampus (coordinates relative to bregma: AP 4 mm, ML 3 mm, and V 2 mm) (Paxinos and Watson, 1986). The electrodes were connected to the multipin socket and were fixed to the skull with dental acrylic cement. EEG recordings were performed 5 days after the surgery.

EEG recordings and analyses

Seizures were monitored in animals by EEG recordings. Rats were allowed to settle for habituation in a Plexiglas cage (25×25×60 cm^3) for at least 20 min, and then connected to the lead socket in a swivel inside a Faraday's cage. Routinely, a 10-min baseline recording was obtained to establish an adequate control period. The effect of oral administration of DTDS on seizure activity induced by KA was investigated by DTDS administration (100 mg/kg, p.o.) or its vehicle (canola oil), 1 h before the injection of KA (10 mg/kg, i.p.). The behavior of the animals was tracked during 4 h, and EEG was concomitantly recorded using a digital encephalographer. All EEG apparatus was obtained from Neuromap EQSA260, Neurotec Ltd., Itajubá, Minas Gerais, Brazil. EEG signals were amplified, filtered (0.1–70.0 Hz, bandpass), digitalized (sampling rate, 256 Hz), and stored in a PC for off-line analysis. Digitalized data from basal (10 min) DTDS administration (1 h) and post-KA (4 h) periods were divided in 30 s segments, and a 4 s sample from each segment was used to measure amplitude wave.

Sample preparation for ex vivo analyses

After 4 h of KA administration, rats were decapitated. Brain was quickly removed, kept in ice, and dissected immediately on a cold plate for separation of hippocampus and cerebral cortex. The samples of hippocampus (hippocampal pool of two animals) and cortex were homogenized in 50 mM Tris–HCl, pH 7.4 (1/4, w/v), and centrifuged at 2400×g for 10 min to obtain the low-speed supernatant (S_1). S_1 was used for reactive species (RS), non-protein sulfhydryl (NPSH), glutathione peroxidase (GPx), and Na^+, K^+-ATPase assays, except for carbonyl protein levels.

RS, carbonyl protein, NPSH content, as well as GPx and Na^+, K^+-ATPase activities, were evaluated in cortex and hippocampus of rats exposed to KA, whereas brain regions are not equally injured after KA administration.

Reactive species (RS) measurement. The RS levels were determined in S_1 by a spectrofluorimetric method, using DCHF-DA assay (Loetchutinat et al., 2005). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCHF) is measured for the detection of intracellular RS. To estimate the level of RS, 10 μl of S_1 was added to 2.98 ml of 50 mM Tris–HCl (pH 7.4) and incubated with 10 μl of 1 mM DCHF-DA. The DCHF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 15 min after the addition of DCHF-DA to the medium. The RS levels were expressed as arbitrary units.

Carbonyl protein levels. Carbonyl protein content was assayed by a method based on the reaction of protein carbonyls with DNPH forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm (Reznick and Packer, 1994). Hippocampal and cortex homogenates (1/4, w/v) were prepared in 50 mM Tris–HCl buffer, pH 7.4, without centrifugation. Homogenate was diluted with Tris–HCl buffer, pH 7.4, in a proportion of 1:8. Three tubes containing aliquots of 1 ml of dilution were incubated at 37ºC for 2 h. A volume of 200 μl of 10 mM DNPH dissolved in 2.0 M HCl was added to two of tubes (duplicate). In the third tube was added only 200 μl of 2.0 M HCl solution (blank). Tubes were incubated for 60 min at room temperature in the dark and vortexed every 15 min. After that, 0.5 ml of denaturizing buffer (sodium phosphate buffer, pH 6.8, containing 3% sodium dodecyl sulfate (SDS)), 1.5 ml of ethanol, and 1.5 ml of hexane were added. The mixture was vortexed for 40 s and centrifuged for 15 min at 2400×g. The pellet obtained was separated and washed two times with 1 ml of ethanol:ethyl acetate (1:1, v/v). The pellet was dissolved and mixed in 1 ml of denaturizing buffer solution. Absorbance was measured at 370 nm. Results were reported as carbonyl content (nmol carbonyl content/mg protein).

Determination of non-protein thiols (NPSH) levels. NPSH levels were determined by the method of Ellman (1959). To determine NPSH, S_1 was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded, and free –SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer, pH 7.4, and 10 mM DTNB. The color reaction was measured at 412 nm. NPSH levels were expressed as μmol NPSH/g tissue.

Glutathione peroxidase (GPx) activity. GPx activity in S_1 was assayed spectrophotometrically through the glutathione/NADPH/glutathione reductase system by the dismutation of H_2O_2 at 340 nm (Wendel, 1981). In this assay, the enzyme activity is measured indirectly by means of NADPH decay. H_2O_2 is decomposed, generating oxidized glutathione (GSSG) from reduced glutathione (GSH). GSSG is regenerated back to GSH by the glutathione reductase present in the assay media, at the expense of NADPH. The enzymatic activity was expressed in nmol NADPH/min/mg protein.

Na^+, K^+-ATPase activity. The reaction mixture for Na^+, K^+-ATPase activity assay contained 3 mM MgCl, 125 mM NaCl, 20 mM KCl, and 50 mM Tris–HCl, pH 7.4, in a final volume of 500 μl. The reaction was started by the addition of ATP to a final concentration of 3.0 mM. For obtaining the ouabain-sensitive activity, samples were carried out under the same conditions with the addition of 0.1 mM ouabain. The samples were incubated at 37 °C for 30 min; the incubation was stopped by adding trichloroacetic acid solution (10% TCA) with 10 mM $HgCl_2$. Na^+, K^+-ATPase activity was calculated by subtracting the ouabain-sensitive activity from the overall activity (in the absence of ouabain). Released inorganic phosphate (Pi) was spectrofluorimetrically measured at 650 nm as described by Fiske and Subbarow (1925), and Na^+, K^+-ATPase activity was expressed as nmol Pi/mg protein/min.

Protein quantification. Protein concentration was measured by the method developed by Bradford (1976), using bovine serum albumin as the standard.

Histology

Another subset of animals (n=3 per group) was used for histological analysis. DTDS was administered at the dose of 100 mg/kg, 1 h before the KA (10 mg/kg, i.p.) administration. Control rats were injected with canola oil (p.o.) plus saline (i.p.). After 7 days of KA administration (Liang et al., 2007), the animals were decapitated, and the hippocampus from individual rat was fixed in 10% formalin to histological evaluation. The hippocampus was embedded in paraffin, sectioned at 4 μm, and stained with Cresyl Violet for light microscopy examination. Cresyl Violet staining was performed to observe neuronal loss in the hippocampus induced by KA. Neuron morphology was observed in areas of the CA1, CA2, and CA3. Only neurons with a visible nucleus and with the entire outline of the cell apparent were considered normal.

Statistical analysis

Seizure incidence was statistically analyzed by the χ^2 method and Fisher's exact test. Data of Racine's score were analyzed by the nonparametric Kruskal–Wallis test. Statistical analysis of latency to the seizure onset was performed using one-way analysis of variance (ANOVA), followed by the Newman–Keuls test. *Ex vivo* analyses were performed using two-way ANOVA, followed by the Newman–Keuls test, when appropriate. EEG records were statis-

Table 1. Effect of pretreatment with DTDS on KA-induced seizures in rats

Groups	Seizures (n/N)[a]	Latency (min)[b]	Racine's score[c]
Control	0/8	ns	0
DTDS 50	0/8	ns	0
DTDS 100	0/8	ns	0
KA	10/10	62.51±12.31	4–5 (4.50±0.16)
DTDS 50+KA	4/10	70.12±14.47	3–4 (3.25±0.25)
DTDS 100+KA	2/10*	91.21±5.65**	1–2 (1.50±0.50)***

Rats were pretreated orally with DTDS (50 or 100 mg/kg, p.o.) or canola oil, and after 1 h they received KA (10 mg/kg i.p.).

[a] Number of animals that showed seizure episodes/number of animals per group.

[b] Time to the appearance for the first seizure episode (minutes).

[c] Interval of Racine's score according to scale of five points of severity (mean±SEM are presented between brackets).

* $P<0.05$ versus KA group (χ^2 method and Fischer's exact probability test).

** $P<0.05$ versus KA group (one-way ANOVA).

*** $P<0.05$ versus KA group (Kruskal–Wallis test).

tically analyzed by two-way repeated-measures ANOVA. Data are expressed as means±SEM. Values of $P<0.05$ were considered statistically significant.

RESULTS

DTDS effects on KA-induced seizures

As shown in Table 1, there were no behavioral changes observed in animals treated with vehicle or DTDS (50 and 100 mg/kg, p.o.).

Behavioral analysis revealed that the systemic (i.p.) administration of an excitotoxic dose of KA (10 mg/kg) induced progressive motor alterations, similar to those previously described by Racine (1972). All animals pretreated with canola oil and then given KA showed behavioral seizures (10/10), and Racine's score reached 4.5± 0.16 points. In most of the animals that received canola oil plus KA, the seizure episodes were manifested by clonic seizures in the forelimbs with rearing and/or generalized clonic convulsions associated with loss of balance. The latency for the onset of the first clonic seizure episode induced by KA was 62.51±12.31 min (Table 1).

Pretreatment of rats with DTDS, at the dose of 100 mg/kg (p.o.), was statistically effective in decreasing the appearance of seizures (2/10) induced by KA, as well as in

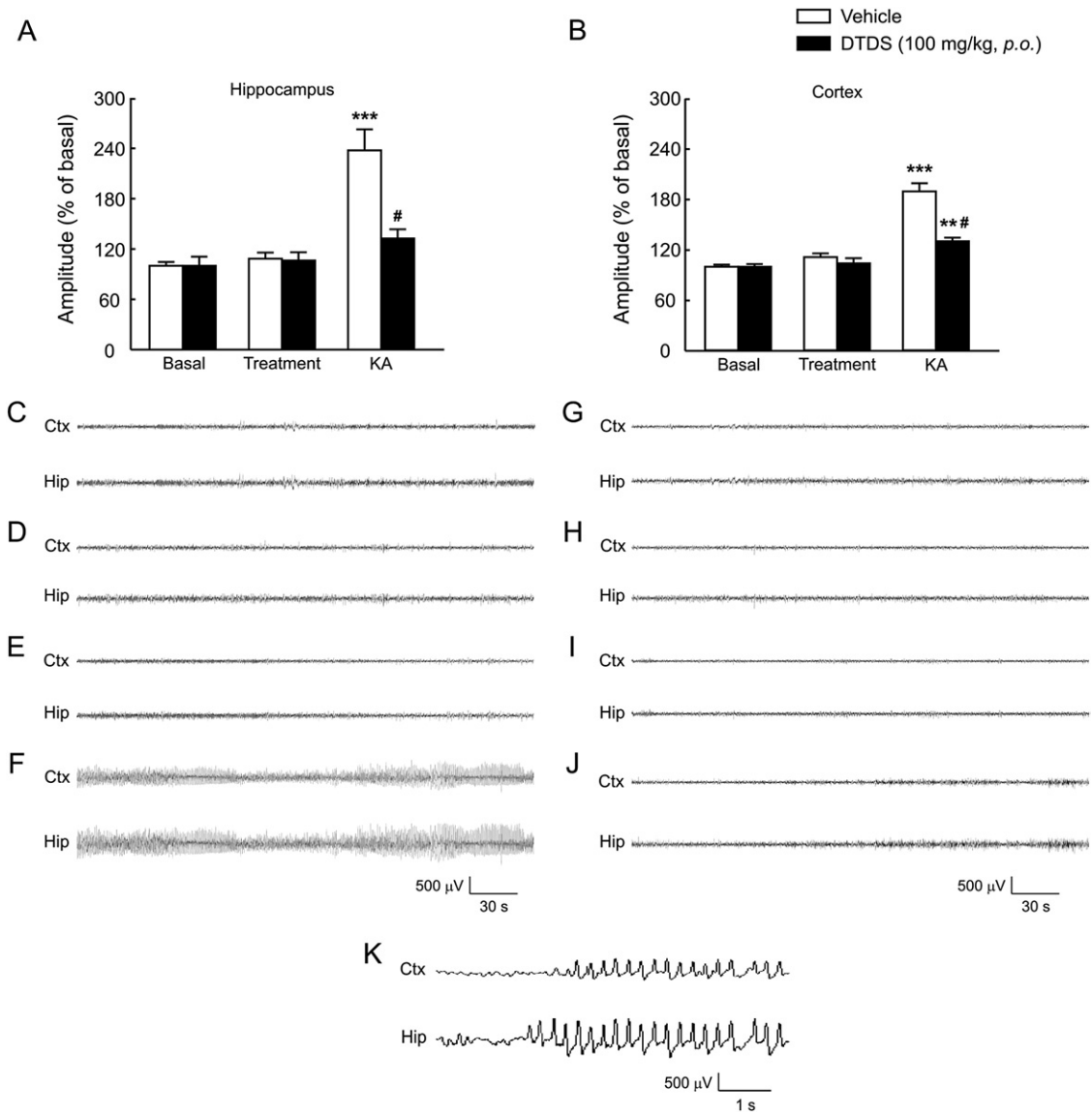

Fig. 2. Effect of pretreatment with DTDS on EEG alterations induced by KA exposure in rat cortex and hippocampus. Rats were pretreated orally with DTDS (100 mg/kg), and after 1 h they received KA (10 mg/kg, i.p.). The behavior of the animals was followedup during 4 h: (A) EEG amplitude in cortex; (B) EEG amplitude in hippocampus (*** $P<0.001$ vs. respective basal, ** $P<0.01$ vs. respective basal, # $P<0.001$ vs. KA, two-way repeated-measures ANOVA, $n=4$ rats/group). (C–J) Representative hippocampal EEG records of rats treated with vehicle (left panel, C–F) and DTDS (right panel, G–J), respectively, where (C) and (G) represent basal records; (D) and (H) represent posttreatment records; (E) and (I) represent records immediately after KA injection; and (F) and (J) represent records 2 h after KA injection; (K) represents the appearance of an epileptogenic focus in the hippocampus induced by KA, as can be evidenced by onset of discharges, with consequent spreading to the cerebral cortex.

Table 2. Effect of pretreatment with DTDS on RS, protein carbonyl and NPSH levels, and GPx activity in hippocampus of rats exposed to KA

Groups	RS[a]	Protein carbonyl[b]	NPSH[c]	GPx[d]
Control	8.69±0.24	1.53±0.04	4.70±0.04	29.48±1.50
DTDS 50	5.33±0.22	1.50±0.06	4.05±0.22	28.00±1.11
DTDS 100	6.10±1.00	1.81±0.32	4.54±0.10	25.80±2.39
KA	31.36±4.91*	2.67±0.08*	3.44±0.61	30.45±1.17
DTDS 50+KA	12.53±3.71**	2.12±0.22**	4.37±0.32	28.81±1.88
DTDS 100+KA	11.89±1.67**	1.74±0.08**	4.71±0.16	30.60±1.31

Rats were pretreated with DDT (50 or 100 mg/kg, p.o.) or canola oil, and after 1 h they received KA (10 mg/kg i.p.).
Data are reported as mean±SEM and expressed as follows: [a] arbitrary units,[b] nmol carbonyl content/mg protein,[c] μmol NPSH/g tissue,[d] nmol NADPH/min/mg protein (two-way ANOVA followed by Student–Newman–Keuls test); n=4–5 hippocampal pool/group (8–10 rats/group).
* $P<0.001$ versus control group.
** $P<0.05$ versus KA group.

increasing the latency for the onset of the first clonic seizure episode (91.21±5.65 min). A decreased Racine's score was observed in the rats that received a 100-mg/kg DTDS pretreatment (1.5±0.50; H(2)=10.59, $P<0.05$). When the animals were pretreated with DTDS plus KA, seizures were attenuated, and most of the animals expressed stereotype mouthing, eye blinking, and/or mild facial clonus, and/or head nodding, and/or severe facial clonus (Table 1).

On the other hand, the lowest dose of DTDS did not show a significant protection against behavioral alterations resulting from KA exposure, although a tendency in reducing KA-induced convulsive behavior was observed (Table 1).

DTDS effects on KA-induced epileptiform EEG activity

As shown in Fig. 2, EEG recordings demonstrated that DTDS, at the dose of 100 mg/kg, did not alter baseline EEG amplitude in rat cortex (Fig. 2A) and hippocampus (Fig. 2B, see also Fig. 2G, H). KA administered at a convulsant dose (10 mg/kg, i.p.) induced EEG seizure activity at both brain structures (Fig. 2A, B). EEG recordings of hippocampus and cerebral cortex revealed that the systemic administration of KA induced the appearance of an epileptogenic focus in the hippocampus, which spread to the cerebral cortex (Fig. 2K). Fig. 2F illustrates hippocampal EEG alterations 2 h after KA administration.

Statistical analysis of quantitative cerebral cortex EEG revealed that DTDS pretreatment, at the dose of 100 mg/kg (p.o.), elicited a significant decrease on EEG alterations (but not back to basal levels) resulting from KA exposure ($F_{(2,12)}$=15.89; $P<0.001$) (Fig. 2A). However, at the dose of 100 mg/kg, DTDS pretreatment completely prevented the increase of EEG amplitude in hippocampus recording, reaching basal measurements, as shown in Fig. 2B ($F_{(2,12)}$=25.19; $P<0.001$) (compare also Fig. 2F, J).

Oxidative stress parameters

RS levels. Two-way ANOVA of hippocampal RS levels revealed a significant DTDS × KA interaction ($F_{(2,21)}$=4.93; $P<0.0175$). Post hoc comparisons revealed that KA produced a significant increase in the hippocampal RS levels in comparison with the control. Preadministration of DTDS, at doses of 50 and 100 mg/kg, protected against the increase of hippocampal RS levels induced by KA. RS levels remained unaltered in the hippocampus of rats that received DTDS (50 and 100 mg/kg) when compared with the control group (Table 2). No alteration in RS levels was observed in cortex of rats exposed to KA and/or DTDS (Table 3).

Carbonyl protein levels. Two-way ANOVA of hippocampal carbonyl levels revealed a significant DTDS × KA interaction ($F_{(2,21)}$=6.58; $P<0.0060$). Post hoc comparisons showed that the exposure of animals to KA significantly increased the carbonyl protein levels in hippocampus when compared with the control group. DTDS, preadministered at the doses of 50 and 100 mg/kg, protected against the increase of the hippocampal carbonyl protein levels caused by KA exposure. DTDS, at both

Table 3. Effect of pretreatment with DTDS on RS, protein carbonyl and NPSH levels, and GPx activity in cortex of rats exposed to KA

Groups	RS[a]	Protein carbonyl[b]	NPSH[c]	GPx[d]
Control	7.60±0.45	1.19±0.05	4.58±0.06	35.01±0.80
DTDS 50	6.49±0.46	1.02±0.09	4.18±0.07	31.07±1.40
DTDS 100	7.06±0.61	1.45±0.10	4.52±0.07	37.77±1.14
KA	7.64±0.51	1.33±0.13	4.22±0.16	38.24±2.24
DTDS 50+KA	8.40±0.19	1.48±0.07	4.41±0.12	38.88±1.39
DTDS 100+KA	8.17±0.33	1.13±0.10	4.55±0.18	34.71±0.96

Rats were pretreated with DTDS (50 or 100 mg/kg, p.o.) or canola oil, and after 1 h they received KA (10 mg/kg, i.p.).
Data are reported as mean±SEM and expressed as follows: [a] arbitrary units, [b] nmol carbonyl content/mg protein, [c] μmol NPSH/g tissue, [d] nmol NADPH/min/mg protein (two-way ANOVA followed by Student–Newman–Keuls test); n=8–10 cortex/group.

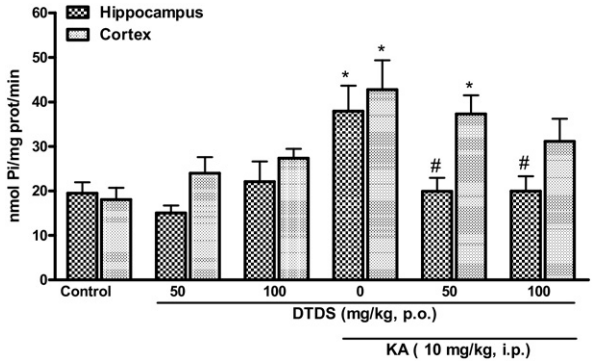

Fig. 3. Effect of pretreatment with DTDS on Na^+, K^+-ATPase activity in hippocampus and cortex of rats exposed to KA. Rats were pretreated orally with DTDS (50 or 100 mg/kg), and after 1 h they received KA (10 mg/kg, i.p.). The results are reported as mean±SEM and expressed as nmol Pi/mg prot/min. # $P<0.05$ versus respective control group * $P<0.05$ versus respective KA group by two-way ANOVA followed by Student–Newman–Keuls.

doses, did not alter hippocampal carbonyl levels when compared with the control group (Table 2). In addition, neither KA nor DTDS treatment altered carbonyl levels in cortex of rats (Table 3).

NPSH levels and GPx activity. There were no alterations on NPSH levels and GPx activity in the hippocampus (Table 2) and cortex (Table 3) of animals exposed to KA and/or DTDS.

Na^+, K^+-ATPase activity. Two-way ANOVA of hippocampal Na^+, K^+-ATPase activity demonstrated a significant DTDS × KA interaction ($F_{(2,21)}=3.61$; $P<0.0450$). Post hoc comparisons showed an increase in hippocampal Na^+, K^+-ATPase activity in the KA group in comparison with the control group. DTDS, at the doses of 50 and 100 mg/kg, was effective in protecting against Na^+, K^+-ATPase activity alterations resulting from KA exposure (Fig. 3).

Statistical analysis of cortical Na^+, K^+-ATPase activity showed a significant main effect of KA ($F_{(1,48)}=13.50$; $P<0.006$). Post hoc comparisons revealed that KA administration to rats increased Na^+, K^+-ATPase activity in cortex when compared with the control group. Pretreatment of animals with DTDS (50 and 100 mg/kg) was not effective in protecting against the increase of cortical Na^+, K^+-ATPase activity caused by KA exposure, although an attenuation of Na^+, K^+-ATPase activity in the animals treated with DTDS, at the highest dose, can be observed (Fig. 3).

As shown in Fig. 3, DTDS administration (50 and 100 mg/kg) did not modify Na^+, K^+-ATPase activity in hippocampus and cortex of rats when compared with the control group.

DTDS effects on KA-induced histopathological changes

Histological analysis of hippocampus carried out under Cresyl Violet staining from control animals exhibited clearly visible neuronal layers. Seven days after KA injection, intense neuronal loss (cellular depopulation) was observed in CA1 (pyramidal), CA2, and CA3 regions from animals exposed to KA, although neuronal loss was more pronounced in the CA3 region. Hippocampi of KA-treated rats showed gliosis process, evidenced by an intense glial cellularity. Pretreatment with DTDS, at the dose of 100 mg/kg, 1 h before KA administration was effective in attenuating hippocampal neurodegeneration (Fig. 4).

DISCUSSION

Behavior and EEG evidence found in this study demonstrated that DTDS relieved KA-induced seizures in rats. Besides, our data showed that DTDS was effective in preventing the hippocampal alterations of oxidative status and Na^+, K^+-ATPase activity. DTDS was also able in attenuating neuronal loss caused by KA exposure.

Organoselenium compounds have highly lipophilic nature (Nogueira and Rocha, 2010), leading us to infer the brain as a potential target for their action. In this context, diphenyl diselenide, an organoselenium compound, has been proven to quickly cross the blood–brain barrier of rodents (Prigol et al., 2010). In the present study, we showed that DTDS (100 mg/kg, p.o.) protected against KA-induced behavioral seizures in rats, providing evidence that DTDS can affect brain processes. Electrographically recorded seizures resulting from KA were characterized by the appearance of an epileptogenic focus in hippocampus that spread to the cerebral cortex. Our EEG findings are in accordance with the literature data, which have shown that the hippocampus is closely related to seizure onset (Liu et al., 2001). Besides, EEG revealed that previous DTDS administration significantly protected against KA-induced seizures in hippocampus, whereas in cerebral cortex, this reduction was partial.

The EEG findings seem to be related to those found for the Na^+, K^+-ATPase activity. DTDS (100 mg/kg) totally protected against the increase in the hippocampal Na^+, K^+-ATPase activity resulting from KA seizures, and there was a tendency of protection in cerebral cortex of rats. Na^+, K^+-ATPase is responsible for the maintenance of ionic gradient necessary for neuronal excitability (Freitas et al., 2003). Similar to investigators who found an increased activity of Na^+, K^+-ATPase as a result of the exposure of rodents to convulsants (Sztriha et al., 1987; Kinjo et al., 2007), we observed that KA stimulated the Na^+, K^+-ATPase activity in cortex and hippocampus. A plausible explanation for this fact is that activation of Na^+, K^+-ATPase could represent a compensatory mechanism in an attempt to control the brain excitability. It has been demonstrated that discharges produced by electrical stimulation are also followed by an increase in the Na^+, K^+-ATPase activity (Bignami et al., 1966; Harmony et al., 1968). Furthermore, the activation of glutamate receptors induces a Ca^{2+}-mediated activation of calcineurin, which dephosphorylates and activates the Na^+, K^+-ATPase (Monfort et al., 2002). In addition, nitric oxide, carbon monoxide, and metabotropic and N-methyl-D-aspartate (NMDA) glutamate receptors also modulate neuronal Na^+, K^+-ATPase activity by activating protein kinases C and G (Nathanson et al., 1995) in a complex phosphorylation cascade of regulatory

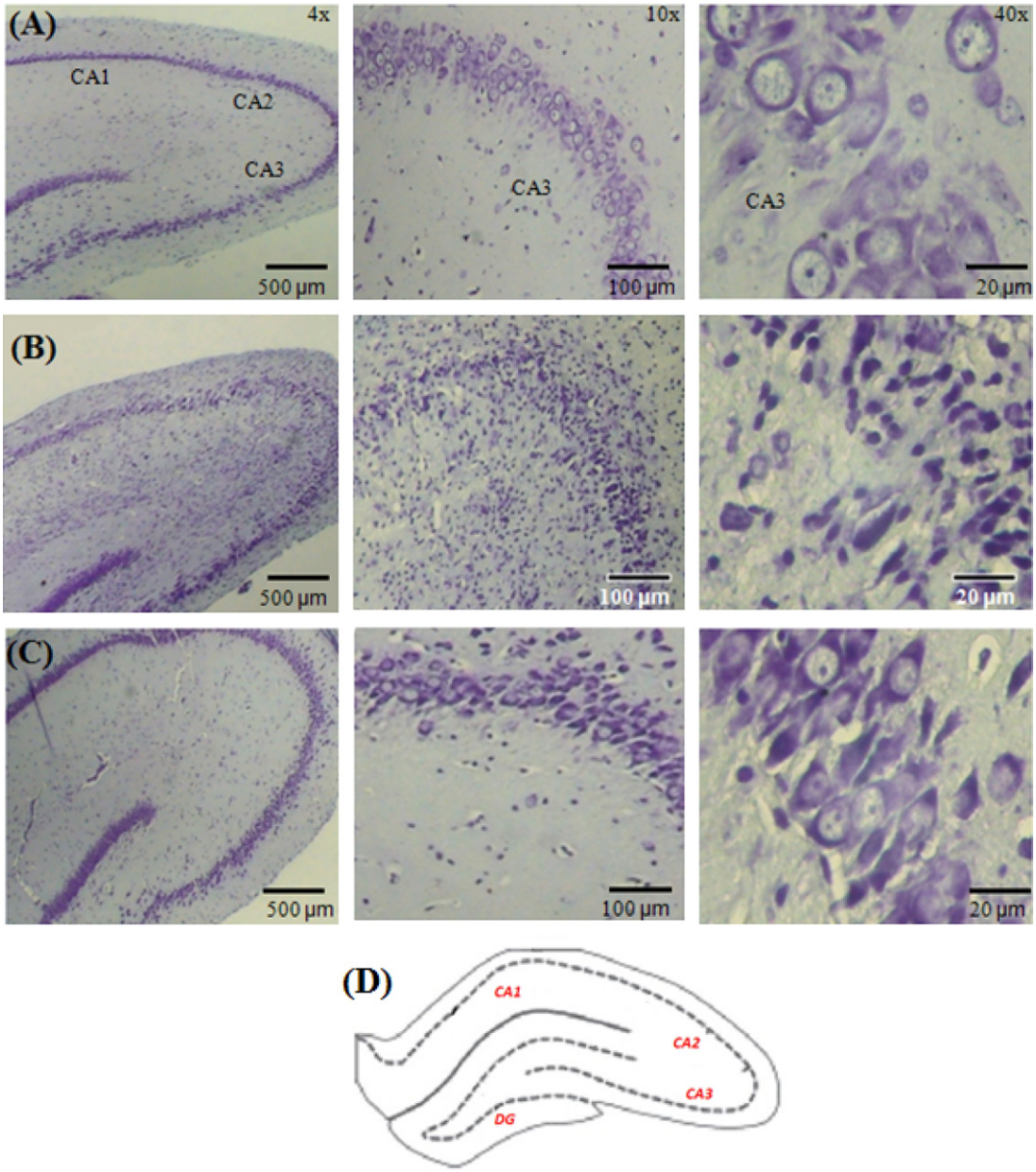

Fig. 4. Photomicrography of histopathological changes in rat hippocampus 7 d after KA exposure. (A) Control group; (B) KA; (C) DTDS, 100 mg/kg plus KA; (D) hippocampal map (CA1, CA2, CA3, dentate gyrus, DG). Wistar rats were pretreated with DTDS (100 mg/kg, p.o.) or canola oil, and the seizures were induced by KA (10 mg/kg i.p.) administration at 1 h after DTDS pretreatment. Cresyl Violet stain: 4, 10, and 40×, respectively. Calibration bars correspond to 500, 100, and 20 μm for augments of 4, 10, and 40×, respectively. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

proteins in which free radicals play a modulatory role (Therien et al., 2001).

Our results revealed that the KA administration induced oxidative damage in rat hippocampus, but not in cerebral cortex, evidenced by increased RS and protein carbonyl levels 4 h after its exposure. The present findings support the hypothesis that the pattern of oxidative injury induced by KA seems to be highly region specific, and the hippocampus is one of the most vulnerable areas to *in vivo* KA-mediated oxidative stress (Candelario-Jalil et al., 2001). Although many epilepsy studies have shown alterations in the antioxidant defenses by KA (Li et al., 2010; Kim et al., 2000; Yalcin et al., 2010), antioxidant defenses analyzed here were not modified. However, we cannot rule out the fact that another antioxidant defense, not evaluated, could be altered by KA exposure.

KA is thought to prosecute its action, at least in part, by increasing glutamate release through presynaptic receptors (Ferkany et al., 1982). In addition to KA receptors, KA also stimulates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptors (Farooqui et al., 2008). It is generally accepted that the overactivation of

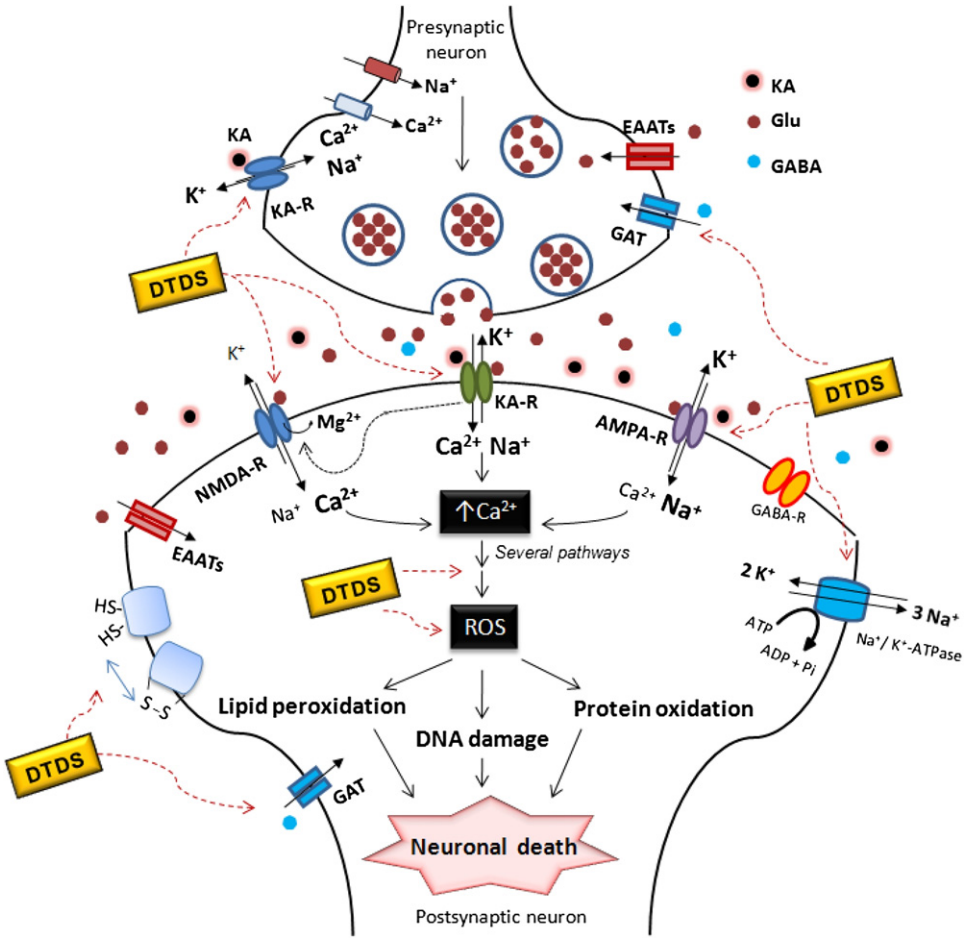

Fig. 5. Schematic presentation of signaling pathways involved in the excitatory amino acid-mediated excitotoxicity and possible targets, which could contribute to anticonvulsant and protective effects of DTDS. KA; Glu, glutamate; NMDA-R, NMDA receptor; KA-R, kainate receptor; AMPA-R, AMPA receptor; EEATs, excitatory amino acid transporters; GAT, GABA transporters.For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

excitatory amino acid receptors triggers a marked intracellular Ca^{2+} rise, leading to the activation of Ca^{2+}-dependent proteases, kinases, and nucleases and the generation of ROS, with consequent neuronal damage (Allison and Pratt, 2003; Wang et al., 2005). Increased ROS levels may be critical in KA excitotoxic effects because ROS can damage lipids, proteins, and DNA and disrupt the membrane integrity, leading to cellular and mitochondrial dysfunctions that are associated with cell death (Lin and Beal, 2006) (Fig. 5).

We observed that DTDS pretreatment prevented the hippocampal oxidative damage resulting from KA injection. Although DTDS could act as an antioxidant in the present study, data of behavioral seizures and EEG seizure point to DTDS anticonvulsant action, thereby reducing the cascade of ROS formation. The results do not allow us to elucidate the exact mechanism by which DTDS caused the anticonvulsant action. However, it is interesting to note that some organochalcogenides are able to modulate neural circuits. It has been reported that acute exposure to ebselen inhibited K^+-stimulated [^{3}H]glutamate release by brain synaptosomes in rats (Nogueira et al., 2002). Prigol et al. (2009) have also demonstrated that *m*-trifluoromethyl-diphenyl diselenide attenuated pentylenetetrazole-induced seizures in mice by inhibiting gamma-aminobutyric acid (GABA) uptake in cerebral cortex slices. Moreover, some receptors and ion channels, such as NMDA receptor, voltage-gated K^+ channels, and GABA-A receptor, are known to be redox sensitive (Ruppersberg et al., 1991). In this context, Nogueira and Rocha (2010) suggest that redox modulation of specific high molecular weight thiol-containing molecules could contribute to the pharmacological effects of the organochalcogens. A schematic presentation of signaling pathways involved in the excitatory amino acid-mediated excitotoxicity and possible targets to DTDS is shown in Fig. 5.

Some authors have shown that systemic or intracerebral injection of KA causes epileptiform seizures in the CA3 region of the hippocampus. These seizures propagate to other limbic structures and are followed by a pattern of cell loss that is similar to those seen in patients suffering from TLE (Nadler, 1981; Ben-Ari, 1985). We demonstrated that DTDS was able to attenuate the neuronal loss in the hippocampal region observed 7 days after KA injection.

Although antioxidant mechanisms could be involved in the DTDS anticonvulsant action, we cannot rule out that the observed reduction of neuronal cell death is secondary to attenuated SE.

In conclusion, the present results showed that DTDS was effective in protecting against neurotoxicity induced by KA. However, the pharmacological mechanisms of anticonvulsant action of DTDS remain to be further elucidated.

Acknowledgments—The authors declare that they have no personal and competing financial interests. The financial support by UFSM, CAPES, CNPq (PIBIC), FAPERGS/CNPq (PRONEX) research grant number 10/0005-1 and FAPERGS research grant number 10/0711-6 is gratefully acknowledged.

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(Accepted 15 July 2011)
(Available online 27 July 2011)