Rosmarinic acid exerts a neuroprotective effect in the kainate rat model of temporal lobe epilepsy: Underlying mechanisms

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Abstract

Context: Temporal lobe epilepsy (TLE) is an intractable neurological disorder. Rosmarinic acid (RA) is a natural polyphenol with antioxidant, anti-apoptotic, and anti-inflammatory properties. Objective: This study evaluates beneficial effect of RA in intrahippocampal kainate-induced model of TLE.

Materials and methods: Rats were divided into sham, RA-pretreated sham, kainate, and sodium valproate (VA) or RA-pretreated kainate groups. Rats received RA or VA p.o. at doses of 10 or 300 mg/kg/d, respectively, starting 1 week before the surgery. After 6 weeks, seizure intensity, apoptosis, and oxidative stress markers were evaluated in addition to determination of Timm index as an indicator of mossy fiber sprouting (MFS) and the number of Nissl-stained neurons. Results: All rats in the kainate group had seizure and 24.3% of rats in the kainate-VA group and 36.7% of rats in the kainate-RA group showed seizure. The kainate group had a significant elevation of malondialdehyde (MDA) (p < 0.05) and nitrite (p < 0.01) and reduction of glutathione (GSH) and catalase activity (p < 0.05) and pretreatment of kainate-lesioned rats with RA or VA significantly lowered MDA and nitrite content (p < 0.05) and raised activity of catalase (p < 0.05). The kainate group also had a significant reduction of neurons in CA1 and CA3 regions and an elevation of Timm index (p < 0.05–0.001) and RA or VA significantly (p < 0.05–0.01) prevented these changes.

Discussion and conclusion: RA could attenuate seizure, mitigates oxidative stress, augments the activity of defensive systems, and prevent hippocampal neuronal loss and MFS in the kainate model of TLE.

Introduction

Temporal lobe epilepsy (TLE) is a chronic neurological condition which is associated with chronic seizures. In this type of epilepsy, seizures have a potential to arise from one or both temporal lobes of the brain (Engel, 2001). TLE occurs in about 30% of epileptic patients, leading to some deficits which remain uncontrolled by surgical removal of epileptic focus or by the usage of antiepileptic drugs. Extensive reorganization of hippocampal circuits, neurodegeneration, and hippocampal sclerosis are some neuropathological hallmarks associated with TLE (Xie et al., 2011). Kainic acid (KA) is an agonist for a subtype of ionotropic glutamate receptor. Limbic seizures can be induced in rats using KA, causing oxidative stress and mitochondrial dysfunction in many regions of the brain, particularly in limbic structures (i.e., hippocampal CA1 and CA3, and the hilus of dentate gyrus) (Lee et al., 2008), leading to neurodegeneration in hippocampal CA3 pyramidal cells (Kim et al., 2012) followed by sprouting of the mossy fibers (Wu et al., 2009) and distinct apoptosis (Chen et al., 2014).

Rosmarinic acid (RA) (3,4-dihydroxyphenyllactic acid) is an ester of caffeic acid. Species of Boraginaceae and the subfamily Nepetoideae of the Lamiales are rich in rosmarinic acid (Petersen, 2003). It has been reported that RA exerts a number of biological and anti-pathological activities. It works as an anti-fibrosis agent in liver and as an antisepsis (Jiang et al., 2011), astringent, antioxidant, anti-inflammatory, and hepato- and cardio-protective chemical. Its anti-inflammatory effect is via inhibition of the enzymes lipoxigenase and cyclooxygenase (Tepe, 2008). RA effectively inhibits oxidative stress-mediated damage in macrophages and astrocytes. In addition, RA has an anti-apoptotic role in cardiac muscle cells, astrocytes, and PC12 cells (Ozturk, 2010). The neuroprotective effect of rosmarinic acid seems to be linked to its antioxidant/radical scavenging properties and its ability to modulate some of the intracellular cascade events leading to neuronal death (Fallarini et al.,
2009). As many TLE patients remain unresponsive to current therapies, natural drugs with fewer side effects can be used for this purpose. Therefore, we decided to investigate the effect of RA in TLE rats.

Materials and methods

All experiments were performed on adult male Wistar rats (250–300 g; n = 60). They were housed three to four per cage in a temperature-controlled colony room under light/dark cycle with food and water available ad libitum. The protocols for animals use and care were approved by the ethics committee of Tehran University of Medical Sciences (IR.TUMS.Z.REC.1391.598) and conducted in compliance with NIH guidelines.

Experimental procedure

Rats were randomly assigned to six groups, i.e., sham, sham + RA, kainate, kainate + valproic acid (kainate + VA) as a positive control, and kainate + RA. For intrahippocampal injections, rats were anesthetized with chloral hydrate (300–350 mg/kg; i.p.), placed into the stereotactic frame (Stoelting Co., Wood Dale, IL) with the incisor bar set at 3.3 mm below the interaural line. The dorsal surface of the skull was exposed and a burr hole was drilled using the following coordinates according to the stereotaxic atlas (Paxinos & Watson, 1986) with the bregma point as the reference: anteroposterior, 4.1 mm; lateral, 4.2 mm; and ventral to the dura, 4–4.2 mm. Freshly prepared kainate (kainic acid; Sigma-Aldrich, St. Louis, MO) solution (5 μL of normal saline containing 1 μg of kainate) was injected into the right side of the hippocampus at a rate of 1 μL/min using a Hamilton microsyringe (Stoelting Co., Wood Dale, IL). The syringe was slowly withdrawn and the scalp was sutured. The sham group received the same volume of normal saline. RA (Sigma-Aldrich, St. Louis, MO) at a dose of 10 mg/kg/d was dissolved in propylene glycol and administered p.o. through a gavage needle for 1 week, with the last injection 1 h pre-surgery. The dose of RA was chosen from a previous study on its protective effect in the brain of streptozotocin-induced diabetic rats (Mushtaq et al., 2014). Valproic acid (VA; Sigma-Aldrich, St. Louis, MO) at a dose of 300 mg/kg/d (Li et al., 2013) was dissolved in normal saline and administered p.o. for 1 week before the surgery.

Behavioral assessment of seizures

All animals were assessed for kainate-induced seizure activity during a 4-h period after the surgery according to Racine’s classification: 0, no reaction; 1, stereotypic mounting, eye blinking, and/or mild facial clonus; 2, head nodding and/or multiple facial clonus; 3, myoclonic jerks in the forelimbs; 4, clonic convulsions in the forelimbs with rearing; and 5, generalized clonic convulsions and loss of balance (Kiasalari et al., 2013; Racine & Chipanshla, 1972).

Assessment of oxidative stress markers

After 6 weeks, some rats (n = 5 for each group) were anesthetized with diethyl ether and decapitated. The hippocampi were isolated and blotted dry, and then weighed and prepared as a 10% tissue homogenate in ice-cold 0.9% saline solution containing protease inhibitor cocktail. After centrifugation (1000 × g, 4°C, 10 min), the supernatant was aliquoted and stored at −70°C until assayed.

Measurement of hippocampal MDA concentration

The concentration of MDA as a marker of lipid peroxidation was determined by measuring thiobarbituric acid reactive substances in the supernatant as described previously (Baluchnejadmojarad & Roghani, 2011).

Determination of hippocampal nitrite concentration

Supernatant nitrite content was assayed by the Griess method according to a previous study (Baluchnejadmojarad & Roghani, 2011).

Reduced glutathione measurement (GSH)

GSH was measured spectrophotometrically as described before (Ellman, 1959; Sediak & Lindsay, 1968).

Assay of catalase activity

For this purpose, Claiborne’s (1985) method was used.

Protein assay

The protein content of the supernatant was measured by the Bradford (1976) method.

Nissl and Timm staining

After 6 weeks, animals (n = 4–5 each group) were deeply anesthetized with ketamine (150 mg/kg) and perfused through the ascending aorta with 50 mL of heparinized normal saline followed by 100 mL of sulfide solution (1.2% Na₂S and 1.0% NaH₂PO₄) and then with 50–100 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Following perfusion, the brains were removed from the skull, and hippocampal blocks were prepared and immersed in 30% sucrose in phosphate buffer at 4°C for 1–2 d. Then, sections were cut at a thickness of 20 μm on a freezing microtome (Leica, Wetzlar, Germany). Every second section was Nissl-stained with 0.1% cresyl violet (Sigma-Aldrich, St. Louis, MO) and alternate sections were used for Timm staining. In Nissl-stained sections, neuronal loss was quantified in CA1 and CA3 regions of the hippocampus in at least four sections for each rat at a level range between −3.6 and −4.3 mm from the bregma using an image capturing and analysis system (Bok Engineering, Monza Monza e Brianza, Italy). The process was repeated at least two times for each section and its average was taken as the final value. Counting was done blind to the treatments received.

Timm staining is an accepted method for the visualization of zinc-containing neuronal elements. Mossy fibers from granule cells in the dentate gyrus undergo reorganization of their terminal projections in epilepsy. To visualize MFS in the inner molecular layer of the dentate gyrus (DG), a modified procedure to label the zinc-containing axons of the granule cells was used (Dariani et al., 2013). The slices were immersed for 5 min in 100% alcohol, 5 min in 70% alcohol, and 10 min in distilled water. The slices were then developed in the dark under continuous agitation for 60 min in Timm
working solution with the following composition: 60 mL of 50% gum Arabic, 10 mL of 2 M sodium citrate buffer (pH 3.7), 30 mL of 5.6% hydroquinone, and 0.5 mL of 17% silver nitrate solution. The staining process was terminated with 2% sodium acetate and the unreacted silver ions were removed with 5% sodium thiosulfate. Assessment of MFS as Timm index was obtained from the absolute value of the area of Timm granules divided by the length of DG. For this purpose, a densitometric method was used. The Timm index for each animal was the mean of four sections. All procedures and analyses were done blind to the treatments.

Statistical analysis

All data were expressed as means ± standard error. For statistical evaluation of non-behavioral data, the parametric one-way ANOVA test was used followed by Tukey's post hoc test. For analysis of behavioral data, the χ² test was used. In all analyses, the null hypothesis was rejected at a level of 0.05.

Results

Seizure activity and behavior

Sham and sham + RA groups showed no signs of seizure activity during the first 24 h post-surgery. In contrast, all rats (100%) in the kainate group exhibited high scores of seizures. In addition, rats injected with KA and pretreated with RA or VA exhibited only mild to moderate behavioral signs (lower seizure scores) as compared with the kainate group. In this respect, only 24.3% of rats in kainate + VA (p < 0.01) and 36.7% of rats in kainate + RA (p < 0.05) showed seizure activity versus the kainate group (Figure 1).

Oxidative stress markers

With respect to markers of oxidative stress (Figure 2), the kainate group showed a significant elevation of the MDA content (p < 0.05) and nitric (p < 0.01) and reduction of GSH (p < 0.05) and activity of the defensive enzyme catalase (p < 0.05) versus the sham group and treatment of kainate lesioned rats with RA or VA significantly lowered MDA and nitric content (p < 0.05) and raised the activity of catalase (p < 0.05) with no significant effect on GSH. Meanwhile, there was no significant changes in the RA-pretreated sham group relative to sham regarding these parameters.

Hippocampus cytoarchitecture in the Nissl staining

In this study, the number of neurons per unit area in the CA1 and CA3 regions was counted and compared among groups (Figure 3). Our results showed that the RA-pretreated sham group did not cause any significant noticeable change in this regard. In contrast, intrahippocampal kainate induced a dramatic and significant degeneration (some neurons had a lower size (slender) and being pyknotic) and reduced the number of neurons in CA1 (p < 0.05) and CA3 (p < 0.005) regions of the hippocampus versus the sham group. In this regard, the neurodegeneration in the hippocampus was demonstrated by cell loss in the dentate hilus and considerable thinning of cell layers in the CA1 and CA3 regions. Meanwhile, the dentate gyrus of the kainate group typically showed granule cell dispersion and displacement and it was 2- to 3-fold broader as compared with the contralateral side (non-injected side) in the upper border. Furthermore, RA or VA pretreatment of the kainate group attenuated these changes (p < 0.05–0.01) as compared with the kainate group. These data suggest that RA or VA pretreatment can protect the neurons against kainate neurotoxicity.

Timm histochemistry

Kainate-induced aberrant MFS was shown by the Timm method 6 weeks post-lesion that selectively labeled synaptic terminals of mossy fibers due to their high zinc content. In the sham groups, little sprouting was present in the DG molecular layer. On the contrary, in the kainate group, Timm staining showed robust MFS that extended into the dentate supra-granular layer and in the RA-pretreated group, supragranular MFS was less intense and more dispersed, although it was still denser than the sham group. We further compared the average width and Timm staining density (as indicated by Timm index) between kainate and RA- or VA-pretreated kainate groups and found that RA or KA pretreatment could significantly reduce MFS width and staining density (p < 0.01). These data indicate that VA or RA pretreatment could prevent kainate-induced aberrant MFS (Figure 4).

Discussion

The KA-induced seizure model is widely used as a standard model of human temporal lobe epilepsy (Kiasalari et al., 2013; Tchekalarova et al., 2013). As a structural analog of glutamate, KA activates excitatory amino acid receptors and triggers neuronal membrane depolarization and increased calcium influx through voltage-dependent calcium channel opened by membrane depolarization following activation of kainate receptors with subsequent induction of the formation of reactive oxygen species (ROS), leading to enhanced oxidative stress (Kanada et al., 2005). In turn, the increased ROS generation leads to dysfunction of mitochondrial respiratory chain and damage to the cell structures, subsequently resulting in neuronal damage (Shih et al., 2004). The brain has an array of antioxidant defensive systems such as catalase, superoxide dismutase, and reduced glutathione.
Figure 2. Malondialdehyde (MDA), nitrite content, catalase activity, and glutathione (GSH) in hippocampal homogenate (n = 5 for each group). RA and VA indicate rosmarinic acid and valproic acid, respectively. *p < 0.05, **p < 0.01 (versus Sham), #p < 0.05 (versus Kainate).
(GSH) to prevent it from over-oxidative damage (Ciftci et al., 2014). Furthermore, oxidative glutamate toxicity is initiated by high concentrations of extracellular glutamate that prevent cysteine uptake into cells, followed by the depletion of intracellular cysteine and the loss of GSH. With a diminishing supply of GSH, there is an accumulation of excessive amount of ROS and ultimately cell death. Behavioral activity is good evidence that antioxidants/free radical scavengers can counteract the neuronal damage induced by KA. There is some evidence that KA-induced neuronal damage was the result of free radicals (Han et al., 2012). RA may alleviate KA-induced excitotoxicity by quenching ROS as well as inhibiting GSH depletion because of the ability of cystine to directly compete with glutamate toxicity. RA could increase the endogenous antioxidant enzymes and glutathione (GSH) and decrease lipid peroxidation in liver tissue under toxic conditions (Yang et al., 2013). In our study, RA pretreatment significantly enhanced catalase activity in hippocampal tissue and this may have produced some of its beneficial effects.

Temporal lobe epilepsy is a chronic and resistant-to-treat neurological disorder hall-marked with recurrent seizures due to the development of recurrent excitatory or inhibitory
circuits. The recurrent seizures are associated with aberrant MFS in dentate region (Epsztein et al., 2005). Kainate injection into the CA3 region of the hippocampus causes the development of epileptic seizures (Baluchnejadmojarad et al., 2013). These seizures are followed by a pattern of cell loss like that seen in patients suffering from TLE (Sperk, 1994). For this reason, kainate-induced brain damage has been routinely used for modeling TLE (Sperk, 1994). Enhanced oxidative stress burden also contributes to kainate neurotoxicity (Shin et al., 2008). A moderate neuronal loss in the CA1 region and a massive neuronal loss in the CA3 region and a typical aberrant MFS into the inner molecular layer were observed following intrahippocampal kainate injection in this study which was consistent with previous studies (Kiasalari et al., 2013). Kainate injection into the hippocampus led to the degeneration of CA3 pyramidal neurons and dentate hilar cells and granule cell axons originating from the DG lose their postsynaptic target cells and sprout into the inner molecular layer. These pathologic changes cause the formation of a functional recurrent excitatory circuit between granule cells that contributes to recurrent seizures (Shetty & Hattiangady, 2007).

Although anti-epileptogenic and anticonvulsant activity of RA has not been previously reported, this study for the first time reports its antiepileptogenic effect. Part of beneficial effect of RA in this study could be attributed to its neuroprotective potential (Fallarini et al., 2009). In this regard, it has been shown that RA is capable of protecting N2A cells against oxidative damage (Ghaffari et al., 2014) and it is also a strong protective agent against 6-hydroxydopamine-induced degeneration of the nigrostriatal dopaminergic system via regulating the ratio of Bcl-2/Bax gene expression that are involved in apoptotic pathway (Wang et al., 2012). Therefore, RA could reduce the detrimental action of neurotoxins and/or excitotoxic agents like kainate on neurons, thus limiting accumulation of extracellular glutamate and preventing apoptotic death of neurons. In our study, due to neuroprotective effect of RA, there were lower degrees of neuronal loss and MFS in the kainate + RA group as compared with the kainate group. In our study, RA pretreatment of the kainate group attenuated oxidative stress burden as was evident by significantly lower levels of MDA and nitrite in hippocampal tissue and this have certainly protected hippocampal neurons against oxidative damage with...
subsequent lower MFS and less severe seizure activity. Anti-apoptotic potential of RA may also be involved in its beneficial effect. Previous reports have shown that in the kainate-induced epileptic seizure model, the protective protein Bcl-2 is down-regulated and hence apoptosis occurs (Zhang et al., 2011) and RA treatment is able to inhibit the apoptotic cascade by increasing Bcl-2 expression (Wang et al., 2012). In this way, RA could prevent kainate-induced apoptotic cell death. In addition, RA exerts a protective effect on astrocytes as shown by their increased viability and decreased apoptosis rate induced by H$_2$O$_2$ through increasing mitochondrial membrane potential and inhibition of caspase-3 activity and attenuation of cellular oxidative stress by decreasing the amount of reactive oxygen species and malondialdehyde (Gao et al., 2005).

Kainate-induced epilepsy also accompanies inflammation with increased production of certain prostaglandins such as prostaglandin E$_2$ following an enhancement in mRNA levels of cyclooxygenase and prostaglandin E$_2$ synthase in the brain tissue and anti-inflammatory agents could reduce the severity of the condition (Ciceri et al., 2002). In parallel, it has been shown that kainate-induced excitotoxicity through induction of matrix metalloproteinases leads to selective neuronal death and neuroinflammation in the hippocampus and inhibitors of such enzymes could attenuate the ensuing neuronal damage and this could be therapeutically relevant in related neurological disorders (Jourquin et al., 2003). In contrast, RA is capable to exert anti-inflammatory effect via attenuation of the expression of nuclear factor-kappaB and tumor necrosis factor-α (Dmitrovic et al., 2014).

In this study, we used VA that is one of the most widely prescribed anti-epileptic drugs as a positive control. Since we administered this drug before intrahippocampal kainate injection, its prophylactic and neuroprotective potential was important in this work. Obtained results clearly showed that RA was potent nearly as much as VA in the kainate rat model of TLE. Given the wide spectrum of convulsant effect of valproate against different seizure types, it has been suggested that valproate acts through a combination of several different mechanisms including the GABAergic system and GABA synthesis, decreasing excitatory synaptic activity through the modulation of postsynaptic non-NMDA receptors, and blockade of voltage-dependent sodium channels in epileptic condition (Elms et al., 2013). Previous reports have also shown neuroprotective potential of valproate in the pilocarpine model of epilepsy (Ezz et al., 2011), attenuation of oxidative stress in its presence (Wang et al., 2013), and its anti-apoptotic effect (Huang et al., 2014).

In conclusion, this study confirms the favorable antiepileptogenic effect of RA via lowering lipid peroxidation and inhibiting hippocampal neuronal loss and aberrant MFS in the kainate-model of TLE. RA benefits should be further studied as adjuvant therapy with other conventional anti-epileptic drugs to reduce their doses and hence, their adverse effects.

**Declaration of interest**

The authors report that they have no conflicts of interest. This work was part of a Ph.D. thesis supported by Tehran University of Medical Sciences (TUMS) in 2012.

**References**


