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Rosmarinic acid is anticonvulsant against seizures induced by pentylenetetrazol and pilocarpine in mice

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ABSTRACT

Epilepsy is a chronic neurological disease characterized by spontaneous recurrent seizures (SRS). Current anticonvulsant drugs are ineffective in nearly one-third of patients and may cause significant adverse effects. Rosmarinic acid is a naturally occurring substance which displays several biological effects including antioxidant and neuroprotective activity. Since oxidative stress and excitotoxicity play a role in the pathophysiology of seizures, we aimed the present study to test the hypothesis that rosmarinic acid displays anticonvulsant and disease-modifying effects. Female C57BL/6 mice received rosmarinic acid (0, 3, 10, or 30 mg/kg; p.o.) 60 min before the injection of pentylenetetrazol (PTZ, 60 mg/kg; i.p.) or pilocarpine (300 mg/kg, i.p.). Myoclonic and generalized tonic–clonic seizure latencies and generalized seizure duration were analyzed by behavioral and electroencephalographic (EEG) methods. The effect of acute administration of rosmarinic acid on mice behavior in the open-field, object recognition, rotarod, and forced swim tests was also evaluated. In an independent set of experiments, we evaluated the effect of rosmarinic acid (3 or 30 mg/kg, p.o. for 14 days) on the development of SRS and behavioral comorbidities in the pilocarpine post-status epilepticus (SE) model of epilepsy. Rosmarinic acid dose-dependently (peak effect at 30 mg/kg) increased the latency to myoclonic jerks and generalized seizures in the PTZ model and increased the latency to myoclonic jerks induced by pilocarpine. Rosmarinic acid (30 mg/kg) increased the number of crossings, the time at the center of the open field, and the immobility time in the forced swim test. In the chronic epilepsy model, treatment with rosmarinic acid did not prevent the appearance of SRS or behavioral comorbidities. In summary, rosmarinic acid displayed acute anticonvulsant-like activity against seizures induced by PTZ or pilocarpine in mice, but further studies are needed to determine its epilepsy-modifying potential.

1. Introduction

Epilepsy is a chronic neurological disease defined by a pathologic and enduring tendency to have recurrent seizures [1]. Importantly, epilepsy is linked to numerous physical, neurological, mental health, and cognitive comorbidities, including heart disease, autism spectrum disorders, Alzheimer's disease, depression, anxiety, and learning and memory deficits [2]. In this context, epilepsy has been considered a major worldwide public health problem [3]. Accordingly, the World Health Organization estimates that epilepsy affects around 70 million people worldwide; 80% of them are in developing countries. In these countries, although most cases can be treated, around 75% of people with epilepsy are not receiving appropriate treatment [4].

Despite the availability of a wide range of antiepileptic drugs (AEDs), about one-third of individuals with epilepsy still experience seizures that do not respond to medication [5]. Development of new therapeutic strategies for seizure activity is therefore important, and understanding the molecular basis of seizure onset and maintenance, as well as probing for new targets for antiepileptic drugs, is a fundamental part of this process. In this context, phytomedicines are important in the development of new antiepileptic drugs, since it has been shown that several plant extracts and products may be useful for the treatment of convulsions or seizures [6].

Rosmarinic acid is an ester of caffeic acids and 3,4-dihydroxyphenyl lactic acid commonly found in a broad range of plant species [7]. A

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number of biological activities have been described for rosmarinic acid, including antioxidant, antiinflammatory, and neuroprotective activity [8,9]. Since oxidative stress [10], inflammatory processes in the brain [11], and excitotoxicity [12] may contribute to the pathophysiology of seizures, it is of particular interest to determine if antioxidant, anti-inflammatory and neuroprotective agents are also anticonvulsant. antiinflammatory, and neuroprotective agents are also anticonvulsant. In this context, a few studies have investigated the effect of rosmarinic acid on seizure activity. For instance, a seven-day pretreatment with rosmarinic acid reduced the severity of kainate-induced seizures in rats [13]. Conversely, in another study, administration of rosmarinic acid did not prevent kindling development induced by pentylenetetrazol (PTZ) in mice [14]. In light of these apparently conflicting results, and in order to further evaluate the anticonvulsant potential of rosmarinic acid, we aimed the present study to investigate the effect of this natural product on seizure activity in experimental models of epilepsy in which it has not been evaluated. In this context, we tested the effect of rosmarinic acid against the seizures induced by acute injection of PTZ or pilocarpine, two convulsants that have been widely used in the preclinical screening of new AEDs [15]. Furthermore, we evaluated the effect of rosmarinic acid on spontaneous recurrent seizures (SRS) and behavioral comorbidities in the pilocarpine-induced status epilepticus (SE) model of epilepsy.

2. Materials and methods

2.1. Animals and reagents

Female C57BL/6 mice (20–30 g; 30–60 days old) were used. Animals were maintained under controlled light and environment (12:12 h light–dark cycle, 24 ± 1 °C, 55% relative humidity) with free access to water and food (Supra™, Santa Maria, RS, Brazil). All experimental protocols aimed to keep the number of animals used to a minimum, as well as their suffering. These were conducted in accordance with national and international legislation (guidelines of the Brazilian Council of Animal Experimentation — CONCEA and of the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals — PHS Policy) and with the approval of the Ethics Committee for Animal Research of the Federal University of Santa Maria.

Pentylenetetrazol (PTZ) and pilocarpine were purchased from Sigma (Sigma-Aldrich, St. Louis, Missouri) and were dissolved in 0.9% NaCl to 6 mg/mL and 30 mg/mL, respectively. Rosmarinic acid was purchased from Sigma and dissolved in 0.9% NaCl containing 5% Tween 80. Doses and schedules for drug injections were selected based on the literature [13,14] and on pilot experiments.

2.2. Acute seizure models

Animals were individually placed in glass boxes and administered, by gavage, with increasing doses of rosmarinic acid (3, 10, or 30 mg/kg) or vehicle (0.9% NaCl containing 0.05% Tween 80). Sixty minutes thereafter, PTZ (60 mg/kg) or pilocarpine (300 mg/kg) was injected intraperitoneally. All solutions were administered at 10 mL per kg of body weight. After the injection of the convulsant, seizure behavior was followed for 15 min (PTZ) or 60 min (pilocarpine). The latency to myoclonic jerks, the latency to generalized seizures, and the duration of the first generalized seizure were recorded.

2.3. Electroencephalography (EEG)

Seizure activity and the effect of rosmarinic acid in acute seizure models induced by PTZ or pilocarpine were evaluated in a subset of animals (n = 3–4) by electroencephalographic (EEG) recordings. The procedures for recording electrode implantation and EEG recordings are described in detail elsewhere [16]. Briefly, a 30-min baseline recording was obtained to establish an adequate control period. After the baseline recording, mice were injected with rosmarinic acid (30 mg/kg, p.o.) or vehicle (10 mL/kg, p.o.), 60 min before the injection of PTZ (60 mg/kg, i.p.) or pilocarpine (300 mg/kg, i.p.). Following convulsant injection, the behavior was monitored, and EEG was recorded for 15 min (PTZ) or 60 min (pilocarpine). The EEG signals were stored in a PC for offline analysis.

2.4. Pilocarpine-induced SE and chronic model of epilepsy

Status epilepticus was induced in C57BL/6 mice following an improved procedure in which repeated low doses of pilocarpine (100 mg/kg, i.p.) are injected until the onset of SE; this ramping protocol has been shown to reduce mortality after SE [17]. Briefly, 30 min before the injections of pilocarpine, methylscopolamine (a muscarinic antagonist) was administered intraperitoneally (1 mg/kg) to reduce adverse peripheral effects. Next, mice were intraperitoneally injected with repeated doses of pilocarpine hydrochloride every 20 min until onset of SE, defined by continuous limbic seizure activity. Status epilepticus was terminated after 60 min with diazepam (10 mg/kg, i.p.). Control animals received methylscopolamine and diazepam but received 0.9% NaCl instead of pilocarpine. All mice were hand-fed with moistened chow and fresh fruits (apples and bananas) and injected with 5% dextrose in lactated Ringer's solution for three days following the SE or control procedure for welfare purposes.

In order to evaluate the effect of rosmarinic acid on SRS and behavioral comorbidities, control and post-SE mice received daily doses of vehicle or rosmarinic acid (3 or 30 mg/kg, p.o.) during 14 consecutive days. Treatment with rosmarinic acid started 3 h after diazepam, and all solutions were prepared fresh daily. Starting 24 h after the SE, all animals were monitored daily for the appearance of SRS. Monitoring was performed during the light phase of the circadian cycle (8–10 h per day) and was carried out until the end of the experimental period (14 days after SE), totaling approximately 100–120 h per animal. In addition, all SRS that occurred during handling (weighing, gavage, tagging, etc.) were noted.

2.5. Behavioral tests

The effect of rosmarinic acid on mice behavior or behavioral comorbidities of epilepsy was evaluated using two different protocols. The sequence of behavioral tests was organized from the least to the most aversive [16].

Protocol #1: In this set of experiments, an anticonvulsant dose of rosmarinic acid (30 mg/kg) was administrated in naïve mice 60 min before each test (open-field, object recognition, rotarod, and forced swim). Independent groups were used in each test, and each animal was used only once.

Protocol #2: Starting one week after the induction of SE, control and SE mice were subjected to a behavioral test battery. All mice underwent all behavioral tests in the following sequence: open-field, object recognition, rotarod, sucrose preference, and forced swim.

The procedures for open-field, object recognition, and forced swim tests are described in detail elsewhere [15]. The rotarod test for naïve animals was carried out as reported in [16], but the paradigm was different for post-SE animals (cutoff latency was 300 s, and the procedure included an additional training session). For the sucrose preference, test mice were placed in individual cages which gave access to two bottles, one with water (100 mL) and the other with a 4% aqueous sucrose solution (100 mL). Water consumption and sucrose consumption over 48 h were measured, and sucrose preference was calculated as a percentage of the total fluid consumed.

2.6. Statistical analyses

Kolmogorov–Smirnov test was used to verify data normality, and Bartlett's test was used to verify homogeneity of variances. Nonparametric data (seizure latencies) were analyzed by Kruskal–Wallis test