Neuropharmacology 67 (2013) 455–464

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Triterpene 3β, 6β, 16β trihidroxilup-20(29)-ene protects against excitability and oxidative damage induced by pentylenetetrazol: The role of Na^+,K^+-ATPase activity

Iuri Domingues Della-Pace [a], Leonardo Magno Rambo [b], Leandro Rodrigo Ribeiro [b], André Luis Lopes Saraiva [b], Sara Marchesan de Oliveira [b], Cássia Regina Silva [b], Jardel Gomes Villarinho [b], Mateus Fortes Rossato [b], Juliano Ferreira [b], Leandro M. de Carvalho [h], Fernanda de Oliveira Lima [h], Ana Flavia Furian [d], Mauro Schneider Oliveria [d], Adair Roberto Soares Santos [f], Valdir A. Facundo [g], Michele Rechia Fighera [e], Luiz Fernando Freire Royes [c,*]

[a] Centro de Ciências da Saúde, Laboratório de Bioquímica do Exercício (BioEx), Programa de Pós-Graduação em Farmacologia – Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil
[b] Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil
[c] Centro de Educação Física e Desportos, Departamento de Métodos e Técnicas Desportivas, Programa de Pós Graduação em Educação Física, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil
[d] Centro de Ciências Naturais e Exatas, Laboratório de Neurotoxicidade, Departamento de Química, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil
[e] Centro de Ciências da Saúde, Departamento de Neuropsiquiatria, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil
[f] Centro de Ciências Biológicas, Laboratório de Neurobiologia da Dor e Inflamação, Departamento de Ciências Fisiológicas, Universidade Federal de Santa Catarina, Trindade, Florianópolis 88040-900, SC, Brazil
[g] Departamento de Química, Universidade Federal de Rondônia, Porto Velho 78900-500, RO, Brazil
[h] Departamento de Química, Universidade Federal de Santa Maria, Caixa Postal 5051, CEP 97110-970 Santa Maria, RS, Brazil

ARTICLE INFO

Article history:
Received 29 March 2012
Received in revised form
24 October 2012
Accepted 26 October 2012

Keywords:
Triterpene
Seizures
Na^+,K^+-ATPase
Oxidative damage
Pentylenetetrazol
Ouabain

ABSTRACT

Administration of the compound triterpene 3β, 6β, 16β-trihidroxilup-20(29)-ene (TTHL) resulted in antinociceptive activity in several pain models in mice. Because pain and epilepsy have common mechanisms, and several anticonvulsants are clinically used to treat painful disorders, we investigated the anticonvulsant potential of TTHL. Behavioral and electrographic recordings revealed that pretreatment with TTHL (30 mg/kg; i.g.) increased the latencies to the first clonic seizure to the tonic–clonic and reduced the duration of the generalized seizures induced by the $GABA_A$ receptor antagonist PTZ (80 g; i.p.). The TTHL pretreatment also protected against PTZ-induced deleterious effects, as characterized by protein carbonylation, lipid peroxidation, [^{3}H] glutamate uptake and the inhibition of Na^+,K^+-ATPase (subunits α_1 and α_2/α_3). Although TTHL did not exhibit DPPH, ABTS radical scavenging activity *per se* and does not alter the binding of [^{3}H]flunitrazepam to the benzodiazepinic site of the $GABA_A$ receptor, this compound was effective in preventing behavioral and EEG seizures, as well as the inhibition of Na^+,K^+-ATPase induced by ouabain. These results suggest that the protection against PTZ-induced seizures elicited by TTHL is due to Na^+,K^+-ATPase activity maintenance. In fact, experiments in homogenates of the cerebral cortex revealed that PTZ (10 mM) reduced Na^+,K^+-ATPase activity and that previous incubation with TTHL (10 μM) protected against this inhibition. Collectively, these data indicate that the protection exerted by TTHL in this model of convulsion is not related to antioxidant activity or GABAergic activity. However, these results demonstrated that the effective protection of Na^+,K^+-ATPase elicited by this compound protects against the damage due to neuronal excitability and oxidation that is induced by PTZ.

1. Introduction

Epilepsy is a neurological disorder characterized by behavioral and electroencephalographic (EEG) changes in a group of neurons in the central nervous system (CNS) (Dichter et al., 2007). This neurological disorder consists of a large group of neurological

* Corresponding author. Fax: +55 55 3220 8031.
E-mail address: nandoroyes@yahoo.com.br (L.F.F. Royes).

0028-3908/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuropharm.2012.10.022

diseases with incidence of 0.5–1% in the general population (Andrade and Minassian, 2007). Our understanding of the pathophysiology of epilepsy has advanced dramatically in last 30 years, particularly in terms of its cellular physiology and genetic bases (Meldrum and Rogawski, 2007). Conversely, although single-drug therapies provide optimal seizure control in approximately 80% of all patients, seizure activity remains uncontrolled in a significant number of individuals, regardless of the type of therapy (Dichter et al., 2007).

From this point of view, several studies have suggested that a cascade of biological events, including overstimulation of the glutamatergic system, understimulation of the GABAergic system, and oxidative stress on selected targets as Na^+,K^+-ATPase, underlies the development and propagation of epilepsy (Patsoukis et al., 2005; Souza et al., 2009). This finding is particularly important, considering that this ion-motive pump plays a key role in regulating and controlling nerve excitability (Vasilets and Schwarz, 1993). Based on the hypothesis that these selected targets are involved in epilepsy, an alternative approach to treatment of this disorder would be to use in order to prevent or slow the progression of seizures.

There are several mechanisms shared by epilepsy and pain induction (de Oliveira et al., 2011; Freitas, 2009; King et al., 2011). Of note, some anticonvulsants are used clinically to treat painful disorders (Feuerbach et al., 2009; Mares and Rokyta, 2009). In this context, Longhi-Balbinot et al. (2009) have demonstrated that triterpene 3β, 6β, 16β-trihidroxilup-20(29)-ene (TTHL) produced significant and specific antinociceptive action against glutamate-, NMDA- and trans-ACPD-induced nociception without causing any detectable locomotor impairment. These experimental findings suggest that the antinociceptive effect of TTHL is closely related to an effect on the glutamatergic system. Because overstimulation of glutamatergic system is a common mechanism for pain and epilepsy, we decided to investigate the effect of TTHL on PTZ-induced behavioral, electrographic and neurochemical alterations in mice.

2. Materials and methods

2.1. Animals

The experiments were conducted using Swiss mice (25–35 g) maintained in a controlled environment (12:12 h light–dark cycle, 24 ± 1 °C, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water. All of the experimental protocols were developed with the goals of keeping the number of animals used to a minimum and maintaining these animals' wellbeing. All of the experiments were conducted in accordance with national and international standards (i.e., the Brazilian School of Animal Experimentation (COBEA) policy and the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals) and with the approval of the Ethics Committee to the Federal University of Santa Maria (113/2010).

2.2. Placement of cannula and surgical procedures for electrocorticographic recording

To determine whether TTHL protects against convulsions induced by ouabain and PTZ, a subset of animals was anesthetized with ketamine (100 mg/kg; i.p.) and xylazine (30 mg/kg; i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a cannula and a set of electrodes were implanted for the purpose of EEG recording. The guide cannula was glued to a multipin socket and inserted into the right ventricle through a previously opened skull orifice. Two screw electrodes were placed over the right (ipsilateral) and left (contralateral) parietal cortices (coordinates in mm: AP −4.5, L 2.5) along with a ground lead positioned over the nasal sinus. The electrodes were connected to a multipin socket and fixed to the skull with dental acrylic cement.

All of the intracerebroventricular (i.c.v.) injections were performed with a needle (30 gauge) protruding 1 mm below a guide cannula. All drugs were injected during a period of 1 min with a Hamilton syringe, and an additional minute was allowed to elapse before the removal of needle to avoid backflow of drug through the cannula. The procedures for EEG recording were carried out as previously described (Cavalheiro et al., 1992). Briefly, the animals were allowed to habituate to a Plexiglas cage (25 cm × 25 cm × 60 cm) for at least 30 min before the EEG recordings. Animals were subsequently connected to the lead socket that resides inside a Faraday's cage. The EEG recordings were executed using a digital encephalographer (Neuromap EQSA260, Neurotec LTDA, Itajubá, MG, Brazil). EEG signals were amplified, filtered (0.1–70.0 Hz, band pass), digitized (sampling rate 256 Hz) and stored on a PC for off-line analysis.

2.3. Reagents, drug administration protocol and seizure evaluation

All of the reagents were purchased from Sigma (St. Louis, USA). The drug TTHL was isolated from the flowers of *Combretum leprosum* by the Department of Organic Chemistry (Universidade Federal de Rondônia, Brazil) and characterized by spectral analyses (RMN-1H) and (RMN-13C) and by comparison with the spectrum literature data (Facundo et al., 1993); the drug showed a degree of purity greater than 98%. All reagents were dissolved in saline solution (0.9%) with the exception of triterpene, which was dissolved in saline plus DMSO/Tween 80. The final concentration of DMSO and Tween 80 did not exceed 10% and did not cause any effect *per se* (Fig. 1).

To evaluate the effect of TTHL on electroencephalographic, oxidative and neurochemical alterations in the cerebral cortex of mice induced by PTZ, the animals were treated with TTHL (1, 3, 10 or 30 mg/kg) or its vehicle by intragastric gavage (i.g.) (Longhi-Balbinot et al., 2012) 60 min before the systemic administration of PTZ (80 mg/kg; i.p.) (Zandieh et al., 2010). In the present study, we also evaluated the participation of Na^+,K^+-ATPase in electroencephalographic alterations exerted by TTHL in this model of excitotoxicity. For this purpose, a subset of animals was treated with TTHL (30 mg/kg) or its vehicle (i.g.) 60 min before administration of ouabain (1 μg/site; i.c.v) (Bagetta et al., 1995; Doggett, 1975).

The presence of seizures was monitored in all animals by electroencephalographic recordings. A 10-min baseline recording was obtained to establish an adequate control period. After this baseline recording, the animals were observed for the appearance of generalized tonic–clonic convulsive episodes for 20 min (Ferraro et al., 1999); tonic–clonic convulsive episodes were defined by generalized whole-body clonus involving all four limbs and the tail, rearing, wild running and jumping, followed by sudden loss of upright posture and autonomic signs, such as hypersalivation and defecation. During the 20-min observation period, the latency for generalized tonic–clonic convulsions was measured. The EEG recordings were visually examined for seizure activity, as defined by the occurrence of the following alterations in the recording leads (McColl et al., 2003): isolated sharp waves (≥1.5× baseline); multiple sharp waves (≥2× baseline) in brief spindle episodes (≥1 s and ≤5 s); multiple sharp waves (≥2× baseline) in long spindle episodes (≥5 s); spikes (≥2× baseline) plus slow waves; multispikes (≥2× baseline, ≥3 spikes/complex) plus slow waves; and major seizure (repetitive spikes plus slow waves obliterating background rhythm, ≥5 s).

2.4. Ex vivo experiments

2.4.1. Tissue processing for neurochemical analyses

Immediately after the behavioral and electroencephalographical evaluation, animals were sacrificed by decapitation. The brain of each animal was later exposed by the removal of the parietal bone. The cerebral cortex was rapidly dissected on an inverted ice-cold Petri dish and homogenized in cold 30 mM Tris–HCl buffer (pH 7.4). This homogenate was used for determination of thiobarbituric acid-reactive substances (TBARS), glutamate uptake, carbonyl content and Na^+,K^+-ATPase activity.

2.4.2. Determination of Na^+,K^+-ATPase activity

Na^+,K^+-ATPase and its α isoforms were assayed as previously described (Nishi et al., 1999; Rambo et al., 2009). Briefly, the assay medium consisted of 30 mM Tris–HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$ and 50 μg of protein in the presence or absence of ouabain (1 mM); the final volume of

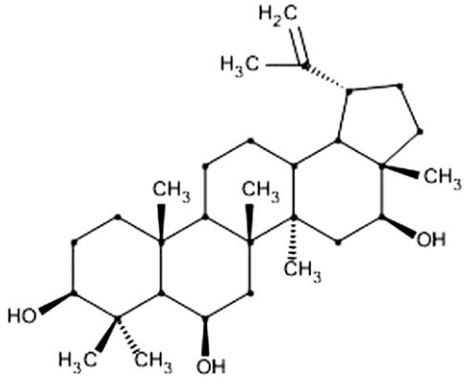

Fig. 1. Molecular structure of triterpene 3β, 6β, 16β-trihidroxilup-20(29)-ene.