Neuroscience 356 (2017) 176–181

CARVACROL PROMOTES NEUROPROTECTION IN THE MOUSE HEMIPARKINSONIAN MODEL

L. M. DATI,[a] H. ULRICH,[b] C. C. REAL,[a] Z. P. FENG,[c] H. S. SUN[c,d] AND L. R. BRITTO[a]*

[a] *Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, Brazil*

[b] *Department of Biochemistry, Institute of Chemistry, University of São Paulo, Brazil*

[c] *Department of Physiology, Faculty of Medicine, University of Toronto, Canada*

[d] *Department of Surgery, Faculty of Medicine, University of Toronto, Canada*

Abstract—Carvacrol is a monoterpene that has been linked to neuroprotection in several animal models of neurodegeneration, including ischemia, epilepsy and traumatic neuronal injury. In this study, we investigated the effects of carvacrol (i.p.) upon the neurodegeneration induced by 6-hydroxy-dopamine unilateral intrastriatal injections in mice. We have also used the cylinder test to assess the behavioral effects of carvacrol in that model of Parkinson's disease, and immunoblots to evaluate the levels of caspase-3 and TRPM7, one of major targets of carvacrol. Behavioral testing revealed that carvacrol largely reduced the asymmetrical use of the forelimbs induced by unilateral 6-hydroxy-dopamine. Carvacrol dramatically reduced the loss of tyrosine hydroxylase immunostaining both in the substantia nigra and in the striatum that are typical of the model. Immunoblots for tyrosine hydroxylase confirmed this effect. Caspase-3 levels were very high after toxin injections, but carvacrol appeared to reduce them to control levels. Finally, TRPM7, observed by immunoblots, increased after 6-hydroxy-dopamine, suggesting the involvement of this cation channel in the ensuing neurodegenerative process. The present data suggest that carvacrol promotes a marked neuroprotection in the 6-hydroxy-dopamine model of Parkinson's disease, possibly by its non-specific blocking effect upon TRPM7 channels.

Key words: carvacrol, dopamine, neuroprotection, TRP channels.

INTRODUCTION

Carvacrol is a naturally occurring monoterpene which has been recently associated to neuroprotection in both *in vivo* and *in vitro* animal models of cerebral ischemia (Yu et al., 2012; Chen et al., 2015a), in a model of epilepsy (Khalil et al., 2017) and in a model of traumatic neuronal injury (Li et al., 2015). Despite the fact that carvacrol has actions in several channels, which include GABA-A and sodium channels, one of its main targets in the context of neuroprotection appears to be the transient receptor potential TRPM7, a cation channel (Parnas et al., 2009; Yu et al., 2012; Oz et al., 2015).

The TRPM7 channel is a member of the transient receptor potential family (Nilius and Owsianik, 2011), specifically of the melastatin subfamily (Fleig and Penner, 2004; Fleig and Chubanov, 2014). This channel is highly permeable for calcium and magnesium ions and contains a kinase domain. There is a myriad of functions associated with TRPM7 and other members of the subfamily, including neural development, neurotransmitter release, and cell proliferation and migration (Sun et al., 2015). In recent years, this channel has been associated with neurodegeneration (Takada et al., 2013; Sun et al., 2015), autophagy (Oh et al., 2015; Sukumaran et al., 2016), tumoral cell proliferation (Sun et al., 2015; Chen et al., 2015b) and neural development (Decker et al., 2014; Turlova et al., 2016), and appears to have a central role in anoxic and ischemic neuronal cell death (Aarts et al., 2003; Sun et al., 2009, 2015; Chen et al., 2015a). There are also reports of TRPM7 involvement in Alzheimer's and Parkinson's diseases (Sun et al., 2015), but this issue is far from being completely understood. It is important to mention that activity levels of TRPM7 channels are regulated by several factors, such as cAMP and pH, and are markedly activated by ATP decrease (Demeuse et al., 2006; Penner and Fleig, 2007) and reactive oxygen species (Dadon and Minke, 2010; Miller and Zhang, 2011). Since oxidative stress is a landmark of Parkinson's and Alzheimer's diseases, it is possible that TRPM7 is activated in these conditions.

This study was undertaken in order to verify if pretreatment with the non-selective TRPM7 inhibitor carvacrol could exert some neuroprotection in 6-hydroxy-dopamine (6-OHDA) model of Parkinson's disease (Schober, 2004), as evaluated by anatomical, biochemical and behavioral approaches. We also evaluated if TRPM7 protein expression is modulated in that model.

*Corresponding author. Address: Dept. of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, 05508-900 São Paulo, S.P., Brazil.
E-mail address: lrbritto@usp.br (L. R. Britto).
Abbreviations: ANOVA, analysis of variance; PFA, paraformaldehyde; SEM, standard error of the mean.

http://dx.doi.org/10.1016/j.neuroscience.2017.05.013
0306-4522/

EXPERIMENTAL PROCEDURES

Animals

Forty-five adult (three months-old) male mice (C57BL/6) were used in this study. The animals were maintained on a 12:12-h light–dark cycle and had free access to food and water. The Institutional Animal Care Committee of the Institute of Biomedical Sciences, University of São Paulo, approved all procedures (Protocol #92/2013), which follow the regulations of the Society for Neuroscience (USA) and the NIH guide for the care and use of laboratory animals (NIH Publications # 8023, revised 1978).

Surgical procedures

The mice were anesthetized with 2–2–2 tribromoethanol (2%, Sigma–Aldrich Co., St. Louis, MO, USA) and placed into a stereotaxic apparatus. 6-OHDA (Sigma Chemical Co., St. Louis, MO, USA) was used at a concentration of 6 μg/μl in saline with 0.1% ascorbic acid. The unilateral injection was performed in the right striatum using a Hamilton syringe (model 701) at the following coordinates: AP: +0.7 mm; ML: 2.0 mm; DV: 3.0 mm relative to the bregma (Paxinos and Franklin, 2001). The total injected volume was 1 μl, and the injection lasted two minutes. The needle was left in place for additional 5 min before it was slowly removed. The control striatum received 1 μl of vehicle (saline in 0.1% ascorbic acid) in the same coordinates. The animals were euthanized for analysis 15 days after the surgery.

Carvacrol treatment

The animals were pretreated with carvacrol (Sigma–Aldrich Co., St. Louis, MO, USA) at a concentration of 40 mg/kg (Chen et al., 2015a). Thirty minutes prior to the injection of 6-OHDA, mice were intraperitoneally (i.p.) injected with carvacrol diluted in saline (NaCl 0.9%).

Immunohistochemistry

Immunostaining was performed as previously described (Hernandes et al., 2013), with small variations. In short, tissue was fixed with 2% paraformaldehyde (PFA) dissolved in 0.1 M phosphate buffer (PB, pH 7.4). The sections were incubated free-floating for 24 h with a mouse anti-tyrosine hydroxylase (Chemicon, Temecula, CA, USA) to detect dopaminergic neurons and terminals, diluted 1:1000 in 0.3% Triton X-100 with 5% normal donkey serum (Jackson ImmunoResearch, PA, USA). A biotinylated secondary antibody (donkey anti-mouse IgG, Jackson ImmunoResearch, PA, USA, 1:200) was then applied to the sections for 2 h. Reactions were developed with avidin–biotin-peroxidase and DAB standard protocols. Immunostaining was analyzed only subjectively based on digital images.

Western blots

Immunoblots were performed as previously described (Hernandes et al., 2013). The membranes were incubated with the following antibodies: mouse monoclonal anti-tyrosine hydroxylase (1:1000; Chemicon, Temecula, CA, USA), goat polyclonal anti-TRPM7 (1:1000; ABCAM, Cambridge, UK), rabbit monoclonal anti-caspase-3 (1:1000; Cell Signaling, Danvers, MA) and a mouse monoclonal anti-β-actin (1:10000; Sigma–Aldrich Co., St. Louis, MO, USA). The probed proteins were developed using a chemiluminescent kit (ECL, Amersham Biosciences, NJ, EUA). Bands were captured with the scanner C-digit (LI-COR, USA). Images were quantified using NIH Image J (USA). Data were always plotted in relation to controls.

Behavioral testing – cylinder test

The cylinder test (Glajch et al., 2012; Smith et al., 2012) was used to test unilateral deficits in voluntary forelimb use and to verify the effects of 6-OHDA and carvacrol upon that behavior. The mice were placed inside a plexiglass cylinder (height 25 cm, diameter 12 cm). Two mirrors were placed behind the cylinder in order to enable a 360° observation of the supporting forepaws in the cylinder walls. The animals stayed in the cylinder for 5 min and their behavior was recorded during this period. No habituation of the mice to the cylinder was allowed before the recordings. The test was performed three times, namely a baseline measurement before the injection of 6-OHDA and 7 and 14 days after the injection. The number of forepaw contacts to the cylinder wall was counted. The score of the cylinder test was calculated as the ipsilateral bias, i.e. the number of ipsilateral forelimb contacts in relation to the total number of forelimb contacts. Healthy rats should score around 50% in the test, and asymmetric animals use more often the ipsilateral forelimb.

Data analysis

Data were expressed as mean ± standard error of the mean (SEM) and were analyzed using a two-way analysis of variance (ANOVA), followed by pairwise comparisons (Tukey's HSD test) where appropriate. The level of significance was established at $p \leq 0.05$.

RESULTS

The carvacrol pretreatment appeared to generate a marked neuroprotection in the 6-OHDA model in immunostaining experiments. (Figs. 1 and 2). Immunostaining for dopamine terminals in the striatum was markedly reduced after 6-OHDA, as expected. Treatment with carvacrol i.p. reduced the loss of terminals in the striatum, as seen in Fig. 1. In the substantia nigra, the loss of dopaminergic neurons after 6-OHDA was also reduced by carvacrol treatment, as clearly seen in Fig. 2. It should be stressed that the results of immunostaining tests were very consistent between experiments (8 animals injected with carvacrol i.p. and 8 animals with saline i.p.).

Immunoblotting for tyrosine hydroxylase confirmed a possible neuroprotection by carvacrol. In the striatum,