Chemico-Biological Interactions 225 (2015) 40–46

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Carnosic acid protects against 6-hydroxydopamine-induced neurotoxicity in *in vivo* and *in vitro* model of Parkinson's disease: Involvement of antioxidative enzymes induction

Chi-Rei Wu [a], Chia-Wen Tsai [b,1], Shu-Wei Chang [c], Chia-Yuan Lin [b], Li-Chun Huang [b], Chia-Wen Tsai [b,*]

[a] The School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University, Taichung, Taiwan
[b] Department of Nutrition, China Medical University, Taichung, Taiwan
[c] Department of Medicinal Botanicals and Health Care, Dayeh University, Changhua, Taiwan

ARTICLE INFO

Article history:
Received 13 August 2014
Received in revised form 25 October 2014
Accepted 14 November 2014
Available online 22 November 2014

Keywords:
Carnosic acid
Neuroprotection
Antioxidative enzymes
Apoptosis
Wistar rats

ABSTRACT

The neuroprotective effects of carnosic acid (CA), a phenolic diterpene isolated from rosemary (*Rosmarinus officinalis*), have been widely investigated in recent years, however, its protection in *in vivo* still unclear. In this study, we investigated the behavioral activity and neuroprotective effects of CA in a rat model of Parkinson's disease (PD) induced by 6-hydroxydopamine (6-OHDA). Rats were treated with 20 mg/kg body weight of CA for 3 weeks before 6-OHDA exposure. Results indicated that CA improved the locomotor activity and reduced the apomorphine-caused rotation in 6-OHDA-stimulated rats. Significant protection against lipid peroxidation and GSH reduction was observed in the 6-OHDA rats pretreated with CA. Pretreatment with CA increased the protein expression of γ-glutamate-cysteine ligase catalytic subunit, γ-glutamate-cysteine ligase modifier subunit, superoxide dismutase, and glutathione reductase compared with 6-OHDA-stimulated rats and SH-SY5Y cells. Immunoblots showed that the reduction of the Bcl-2/Bax ratio, the induction of caspase 3 cleavage, and the induction of poly(ADP-ribose) polymerase (PARP) cleavage by 6-OHDA was reversed in the presence of SB203580 (a p38 inhibitor) or SP600125 (a JNK inhibitor) in SH-SY5Y cells. Rats treated with CA reversed the 6-OHDA-mediated the activation of c-Jun NH2-terminal kinase and p38, the down-regulation of the Bcl-2/Bax ratio, the up-regulation of cleaved caspase 3/caspase 3 and cleaved PARP/PARP ratio, and the down-regulation of tyrosine hydroxylase protein. However, BAM7, an activator of Bax, attenuated the effect of CA on apoptosis in SH-SY5Y cells. These results suggest that CA protected against 6-OHDA-induced neurotoxicity is attributable to its anti-apoptotic and anti-oxidative action. The present findings may help to clarify the possible mechanisms of rosemary in the neuroprotection of PD.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that is caused by a loss of dopaminergic neurons in the substantia nigra. The clinical symptoms of PD are characterized by a combination of bradykinesia, resting tremor, rigidity, and postural instability [1]. The brain in PD is more susceptible to oxidative damage because it is rich in polyunsaturated fatty acids and has high oxygen utilization. Recent studies have suggested that oxidative stress is implicated in the dopaminergic neuronal cell death in PD [2]. Postmortem studies of PD patients reveal increased lipid peroxidation and DNA fragments along with decreased glutathione (GSH) levels in the substantia nigra [3,4]. Excessive reactive oxidative stress (ROS) production and low antioxidant levels lead to cellular protein, lipid, and DNA injury and subsequent cellular apoptosis. Therefore, modulation of intracellular ROS may provide a new approach to the prevention and treatment of PD.

6-Hydroxydopamine (6-OHDA), a potent neurotoxin, is commonly used to generate PD models *in vivo* and *in vitro*. Studies have shown that injection of animals with 6-OHDA destroys nigral dopaminergic neurons, depletes the dopamine neurotransmitter, and increases motor impairment [5,6]. Moreover, 6-OHDA is reported to cause caspase-3 activation and nuclear condensation in rat PC12 cells and human SH-SY5Y cells, which leads to typical apoptotic cell death [5,7]. 6-OHDA causes PD pathogenesis process is related to the generation of excessive ROS. 6-OHDA generates lipid peroxidation, depletes the GSH content, and reduces superoxide dismutase (SOD) activity in the neuron [8]. GSH is a central

* Corresponding author. Tel.: +886 4 2205 3366x7521; fax: +886 4 22062891.
E-mail address: cwtsai@mail.cmu.edu.tw (C.-W. Tsai).
[1] Graduate student.

http://dx.doi.org/10.1016/j.cbi.2014.11.011
0009-2797/

antioxidant and redox modulator in neurons [9,10]. GSH is also the co-substrate for several key cellular antioxidative enzymes, including GSH peroxidase (GPx) and GSH reductase (GSR). Many studies have indicated that GSH depletion leads to oxidative stress induction, mitochondrial complex I inhibition, ubiquitin–proteasome dysfunction, and ultimately neuronal cell death [11,12].

Carnosic acid (CA) is a phenolic diterpene from rosemary (*Rosmarinus officinalis*). Studies have shown that CA inhibits the inflammation induced by phorbol 12-myristate 13-acetate through down-regulation of the mRNA expression of IL-1β and TNF-α in mouse ear [13]. Moreover, CA reduces lipid peroxidation [14] and protects red cells against oxidative hemolysis [15]. Hou et al. (2013) showed that CA protects neuronal cells from ischemia injury through the scavenging of ROS [16]. Additional studies have revealed that CA attenuates dieldrin-induced apoptotic molecules is associated with the production of brain-derived neurotrophic factor [17]. In our previous study, we indicated that CA prevents 6-OHDA-induced cell death in SH-SY5Y cells via mediation of glutathione synthesis and down-regulation of the c-Jun NH2-terminal kinase (JNK) and p38 signaling pathways [18]. Although CA is currently being investigated in the prevention of neurodegenerative disorders, no reported studies have described its neuroprotective role in a 6-OHDA rat model of PD. Therefore, in the present study, we further investigated the extent to which CA protects against oxidative stress and apoptosis in a rat model of PD induced by 6-OHDA.

2. Materials and methods

2.1. Chemical

CA was obtained from A. G. Scientific, Inc. (San Diego, CA). DMEM, penicillin–streptomycin, and trypsin–EDTA were obtained from Gibco Laboratory (Gaithersburg, MD). Fetal bovine serum was from Hyclone (Logan, UT). 6-OHDA, dimethyl sulfoxide (DMSO), triton X-100, Tween 20, β-tubulin, and β-actin were purchased from Sigma Chemical Company (St. Louis, MO). SP600125 and SB203580 were purchased from TOCRIS (Ellisville, MO). BAM7 was purchased from BioVision (Milpitas, CA). Caspase 3, cleaved caspase 3, poly(ADP)-ribose polymerase (PARP), cleaved PARP, Bcl-2, and Bax antibodies were from Cell Signaling Technology (Beverly, MA). γ-Glutamylcysteine ligase catalytic subunit (GCLC) was from Abcam (Cambridge, UK). γ-Glutamylcysteine ligase modifier subunit (GCLM), phospho-JNK, phospho-p38, JNK, p38 and tyrosine hydroxylase (TH) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). GSR and SOD were purchased from GeneTex (San Antonio, TX).

2.2. Animals and treatments

Male Wistar rats, 6–8 weeks old, were obtained from BioLASCO Experimental Animal Center (Taipei, Taiwan). The animals were maintained on 12 h light/12 h dark cycle and 50–70% humidity at 23 ± 1 °C. Animals were housed in groups of four animals per cage with free access to food and water *ad libitum*. For the use of animals in the study, ethical approval was obtained from the Institutional Animal Care and Use Committee of China Medical University (Protocol No. 97-140-N). After 1 week of acclimation, the animals were randomly divided into three groups: vehicle-treated sham-operated controls (sham, $n = 10$), 6-OHDA-treated lesion group (lesion, $n = 11$), and CA-treated 6-OHDA (CA + L, $n = 11$) group. CA was dissolved in 0.5% sodium carboxy methyl cellulose. CA was given at a dose of 20 mg/kg body weight by oral intubation three times each week for 3 weeks before lesioning to rats. After CA pretreatment, 6-OHDA was administered as a single injection in the right striatum. On day 15 of lesioning, all rats were tested for rotational behavior and locomotor activity at room temperature in laboratory without any outside interference. On day 16 of lesioning, rats were anesthetized with carbon dioxide. The striatum and substantia nigra were dissected out quickly for the assays.

2.3. Intrastriatal administration of 6-OHDA

After 3 weeks of treatment with CA, all animals in the experimental group were anaesthetized with intraperitoneal injection of 50 mg/kg tiletamine/zolazepam (Zoletil50®; Virbac Lab., Carros, France). The rats were then placed on a double manipulator stereotaxic frame. The skin was cut to expose the skull. Lesion coordinates were as follows: antero-posterior: +1.5 mm, lateral: −4 mm, dorso-ventral: −7.2 mm. All animals in the experimental group were lesioned by injecting 12.5 μg/2.5 μL 6-OHDA in 0.5% ascorbic acid-saline through the hole into the right striatum, whereas the sham-operated group received 2.5 μL of 0.5% ascorbic acid-saline. The injection rate was 1 μL/min and the needle was kept in place for an additional 1 min before being slowly retracted.

2.4. Apomorphine-induced rotation

The rats were tested for apomorphine-induced rotational behavior on day 15 of lesioning. The animals were given 0.25 mg/kg apomorphine (in 0.5% ascorbic acid-saline) subcutaneously to monitor contralateral rotations. The rotations towards the contra-lateral side were collected at 15 min intervals. The results were expressed in rotations/15 min.

2.5. Locomotor test

The locomotor test was monitored in a computerized TRU Scan™ photobeam sensor E63-12, which consists of a chamber (50 × 50 × 40 cm), an activity monitor, a programmer/processor, and a printer. The activity chamber was furnished with black paper to provide contrast on the screen. On day 15 of lesioning, rats were individually placed in the chamber and assessed for locomotor activity for 15 min each. The data were analyzed for the intervals (min), locomotion (s), rest (s), and distance travelled (cm). The activity chamber was swabbed with 70% alcohol before each use to avoid interference due to animal odors. Results were expressed in terms of activity/15 min.

2.6. Tissue preparation

The animals were sacrificed by carbon dioxide on day 16 of lesioning and their brains were taken out quickly and kept on ice. The right striatum and substantia nigra were homogenized (10% w/v) in 0.1 mol/L potassium phosphate buffer. The homogenate of striatum was then centrifuged at 15,000 rpm for 30 min at 4 °C to obtain supernatant.

2.7. Thiobarbituric acid-reactive substances (TBARS) assay

The method of Khuwaja et al. (2011) was modified for the estimation of lipid peroxidation [6]. The homogenate was pipetted out in glass tubes. Then, 5% trichloroacetic acid and 0.67% thiobarbituric acid (TBA) were added to each tube and mixed thoroughly after each addition. The mixture was centrifuged at 1000*g* for 15 min. The supernatant was transferred to another glass tube and the tube was placed in a boiling water bath for 10 min. After the test tubes were cooled, the absorbance of the color was read at 535 nm using