Neuroscience Letters 606 (2015) 53–59

Contents lists available at ScienceDirect

Neuroscience Letters

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

Research paper

Histopathological and electrophysiological indices of rotenone-evoked dopaminergic toxicity: Neuroprotective effects of acetyl-L-carnitine

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HIGHLIGHTS

- Rotenone evoked dopaminergic neuronal and transporter damage.
- Motor conduction velocity and motor latency were affected by rotenone.
- A co-treatment with acetyl-L-carnitine prevented rotenone induced toxicity.

ARTICLE INFO

Article history:
Received 23 June 2015
Received in revised form 28 July 2015
Accepted 24 August 2015
Available online 28 August 2015

Keywords:
Rotenone
Midbrain
Tyrosine hydroxylase
Dopamine transporter (DAT)
Peripheral nerves

ABSTRACT

Exposure to the natural pesticide, rotenone, a potent mitochondrial toxin, leads to degeneration in striatal nerve terminals and nigral neurons. Rotenone-induced behavioral, neurochemical and neuropathological changes in rats mimic those observed in Parkinson's disease (PD). Here, protective effects of acetyl-L-carnitine (ALC) in the brain dopaminergic toxicity after a prolonged exposure to rotenone were evaluated using electrophysiological and immunolabeling methods. Adult, male Sprague–Dawley rats were injected i.p. with rotenone alone (1 mg/kg) or rotenone with ALC (either 10 or 100 mg/kg; ALC10 or ALC100, respectively) once daily on days 1, 3, 5, 8, 10, 12, 15, 17, 19, 22, 24, 26, 29, 31, 33 and 37. Control rats received either 100 mg/kg ALC or vehicle (30% Solutol HS 15 in 0.9% saline) injections. Animals were weighed on injection days and monitored daily. Motor nerve conduction velocity (MCV) was assessed within two days after treatment using compound muscle action potentials (CMAP) detected from the tail muscle through surface receiver electrodes installed around the distal part of the tail. Rats were perfused immediately after testing with 4% paraformaldehyde and immunohistochemical analysis of dopamine transporter (DAT), tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP), and microglial CD11b marker was performed in the caudate-putamen (CPu) and the substantia nigra pars compacta (SNc) in order to estimate dopaminergic neuronal and transporter damage. Additionally, effects of ALC on preventing microglial or astrocytic hypertrophy were also evaluated. In rats exposed to rotenone and rotenone/ACL10, a significant increases in both proximal (S1) and distal (S2) motor latency and a decrease in MCV were detected in tail nerves ($p < 0.05$). The conduction parameters in rats co-treated with rotenone/ACL100 were not different from control. It was found that 100 mg/kg ALC prevented loss of TH and a decline of DAT level in the midbrain and also prevented the activation of both microglia and astroglia after rotenone treatment. Data indicate neuroprotective effects of ALC in rotenone-evoked dopaminergic neurotoxicity.

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1. Introduction

Scores of studies have investigated the selective dopaminergic toxicity of rotenone in animal models. High-dose, intravenous infusions of rotenone for 7–9 days in rats have been shown to result in selective tissue damage in the striatum and globus

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http://dx.doi.org/10.1016/j.neulet.2015.08.044
0304-3940/Published by Elsevier Ireland Ltd.

pallidus [1]. At lower doses, rotenone alters calcium signaling and can induce oxidative stress leading to oxidative damage in various brain regions [2]. Minipump-mediated continuous intravenous infusions of rotenone for 7–35 days results in selective pathological and biochemical changes in the nigro-striatal dopaminergic system and α-synuclein aggregation (Lewy neurites), which is a characteristic of the early stages of Parkinson's disease (PD) [3].

In a recent study, animals expressing a pathogenic mutant of the α-synuclein protein showed a greatly increased sensitivity to rotenone exposure that was accompanied by significantly impaired mitochondrial function and progressive nigro-striatal damage [4]. Additionally, chronic rotenone intoxication that causes enhanced oxidative and nitrosative stress and induces mitochondrial dysfunction and ultrastructural damage, also results in apoptotic cell death in the striatum *via* a cytochrome c/caspase-3 signaling cascade [5]. The striatal immunoreactivity of TH, an enzyme which catalyzes the rate limiting step in the synthesis of dopamine, was markedly decreased in rats exposed to rotenone [2]. However, this loss of reactivity was not correlated with altered motor behavior in individual rats [1]. Rotenone-induced motor neuron death in the rat spinal cord has been shown in another study, suggesting that spinal cord damage may be linked to degeneration of higher brain nuclei [6].

Motor nerve conduction velocity (MCV) assessments, frequently used in experimental models of peripheral neuropathy [7–10], combined with histopathological analyses prove useful for the characterization of such condition as well as the evaluation of treatments.

Carnitines counteract changes in energy metabolism and decreasing energy production in mitochondrial dysfunction. L-Carnitine and its ester acetyl-L-carnitine (ALC) have been shown to have neuroprotective effects in various conditions of metabolic stress such as ischemia/hypoxia or aging [11]. ALC was able to reduce *in vitro* toxicity induced by blocking mitochondrial complex I with *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPP^+) [12]. The aim of this study was to evaluate neuroprotective effects of ALC in central and peripheral neurotoxicity induced in rats by prolonged exposure to rotenone.

2. Materials and methods

2.1. Animals and treatment

Adult, male Sprague–Dawley rats of the Charles River cesarean delivered (CD) strain 23 weighing on the first day of treatment approximately 270–450 g were used in the study. Animals were kept under controlled environmental conditions (temperature 22 °C, relative humidity 45–55%, 12-h light/dark cycle) and housed two per Plexiglas cage having access to NIH-41 Irradiated Rodent Diet (Harlan Teklad, Madison, WI) chow. Tap water was supplied *ad libitum*. Animal procedures were approved by the NCTR Institutional Animal Care and Use Committee (IACUC) and conducted in full accordance with the Public Health Service policy on humane care and use of laboratory animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996). Rats were randomly allocated to treatment groups. They were injected intraperitoneally (i.p.) with rotenone alone (1 mg/kg) or rotenone with ALC [(Sigma-tau, s.p.a.) either 10 or 100 mg/kg; ALC10 or ALC100, respectively] once daily on days 1, 3, 5, 8, 10, 12, 15, 17, 19, 22, 24, 26, 29, 31, 33, and 37. Control rats received either 100 mg/kg ALC or vehicle (30% Solutol HS 15 in 0.9% saline) injections [13]. Animals were weighed on injection days and monitored daily.

2.2. Electrophysiological testing

MCV was assessed within two days following the last day of exposure to rotenone using compound muscle action potentials (CMAP) detected from the tail muscle through surface receiver electrodes installed around the distal part of the tail as described earlier [7]. Two bipolar surface electrodes (silver wires) were attached 40 mm apart on the upper part of the tail and used to deliver two separate 0.2 msec electrical stimuli of 91 V, 37 μA (proximal S1 and distal S2). Electrical stimulation was triggered through a stimulator (model S48, Grass Medical Instruments, W. Warwick, RI). The associated evoked potential signals were amplified and recorded on the PC. General anesthesia was maintained during the procedure with a Stoelting (Wood Dale, IL) system (2.5% isoflurane and 97.5% oxygen with a flow rate of 5 L/min). The tail surface temperature was maintained during recording at 31.1 ± 0.4 °C. The MCV (m/sec) was calculated using LabVIEW 8.5 (National Instruments) computer software according to the equation d/S1-S2, where d is the distance between stimulating cathodes S1 and S2 and S1-S2 is the motor latency difference. Rats were perfused with 4% paraformaldehyde immediately after the MCV testing.

2.3. Immunohistological analyses

For TH, GFAP, and CD11b immunolabeling, brain sections were initially washed in phosphate buffered saline (PBS) to remove excess fixative and then incubated in 0.5% H_2O_2 for 15 min to quench tissue peroxidase activity. Sections were then briefly rinsed in PBS until bubble formation ceased. Sections were subsequently incubated in Triton-X-100 + PBS for 45 min to permeabilize the tissue to enhance penetration of the antibodies. Sections were then incubated in the same buffered solution containing blocking serum (10% normal horse serum) before they were incubated in polyclonal antibodies against rabbit DAT (1:500; Phosphosolutions Denver, CO), rabbit TH (Calbiochem, CA; 1:1000), rabbit GFAP (DAKO, CA; 1:2000) or rat CD11b (AbD Seroteck,1:1000) for 2 days at 4 °C. Primary antiserum dilutions were made in 1% normal horse serum in the PBS, containing 0.08% sodium azide and 0.02% Kodak Photo-Flo. After thorough rinsing in PBS and Triton-X -100, sections were incubated in a solution of biotinylated donkey anti-rabbit IgG or donkey anti-Rat IgG for 2 h (1:200, Jackson Immunoresearch, PA). The sections were then rinsed in PBS three times and incubated in Streptavidin-peroxidase conjugate (ABC: 1:100 dilution Vector lab, CA) for 1 h at room temperature. Sections were then rinsed in two changes of buffered saline for 5 min each, briefly rinsed in Tris-HCl and developed in DAB (0.03% H_2O_2 + 5 mg DAB), which produced a brown reaction product. The sections were mounted onto gelled slides from distilled water, air dried on a slide warmer at 50 °C for 5 min, cleared in xylene and coverslipped using mounting media DPX. Images of brain tissue slices were captured with a Nikon digital camera (Diagnostic Instruments, MI, USA) using NIS elements and a PC to create individual and composite images for analysis.

3. Statistical analysis

Nerve conduction velocity data were analyzed using one-way ANOVA and multiple comparisons with the Holm-Sidak test. DAT immunoreactivity, TH immunoreactivity, GFAP and CD11b immunoreactivity in the CPU was analyzed using Image J software. Positive pixel intensity method was adopted while doing analysis and then data were subjected to one-way ANOVA followed by Sidak's multiple comparisons test, performed using GraphPad Prism version 6.02 for Windows, (GraphPad Software, La Jolla, CA. USA,). In all cases, a *p*-value less than 0.05 was considered significant.