Neuroscience Letters 552 (2013) 62–65

Contents lists available at ScienceDirect

Neuroscience Letters

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

Systemic diphenidol reduces neuropathic allodynia and TNF-alpha overexpression in rats after chronic constriction injury

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HIGHLIGHTS

- Diphenidol effectively inhibited CCI-evoked allodynia in rats.
- Diphenidol dose-dependently restored lower paw withdrawal threshold in CCI rats.
- Higher dose of diphenidol reduced TNF-α expression in the sciatic nerve of CCI rats.

ARTICLE INFO

Article history:
Received 5 April 2013
Received in revised form 17 June 2013
Accepted 21 July 2013

Keywords:
Diphenidol
Intraperitoneal injection
Mechanical allodynia
Tumor necrosis factor-α
Chronic constriction injury

ABSTRACT

Diphenidol has been shown to block voltage-gated Na^+ channels, which are associated with specific types of pain. Here, we evaluated the effects of diphenidol on chronic constriction injury (CCI)-evoked allodynia and expression of tumor necrosis factor-α (TNF-α). A peripheral nerve injury was elicited in rats by placing four loosely constrictive ligatures around the sciatic nerve. After intraperitoneal injection of diphenidol, rats were tested for evidence of mechanical allodynia prior to surgery, and on postoperative days 3, 6, 7, 11, 13 and 14. We showed that CCI rats received diphenidol caused dose-dependent increases in mechanical withdrawal threshold. Both diphenidol 2 and 10 μmol/kg groups, but not 0.4 μmol/kg diphenidol, displayed lower TNF-α level in the sciatic nerve than the CCI group ($P < 0.05$) on day 7 after CCI. Our results support the conclusion that systemic diphenidol produced a dose-related inhibition of mechanical allodynia following chronic constriction injury of the sciatic nerve. This antiallodynic effect is related to the decrease of TNF-α expression in the sciatic nerve of CCI rats.

Diphenidol is a nonphenothiazinic antiemetic drug widely applied to treat the patients with labyrinthopathies and Meniere's disease in the Latin American, where it is also administered rectally, parenterally, and orally [19] for the management of apomorphine-induced vertigo and vomiting [8,14,28]. Recently, it has been shown that diphenidol produced spinal anesthesia and suppressed voltage-gated K^+, Ca^{2+}, and Na^+ currents [15,16]. There is a growing body of evidence that Na^+ channel blockers attenuate neuropathic pain in rat animal models [1,11,21,22,29]. We suggested that the Na^+ channel blocker diphenidol might involve the block of neuropathic pain.

Pro-inflammatory cytokines (e.g., tumor necrosis factor-α) can cause pain [12,24]. By comparison, therapies with pro-inflammatory or anti-inflammatory cytokine inhibitors decrease pain [24,26]. Furthermore, in activated macrophages and glial and Schwann cells varying responses of local inflammation and over-expression of activated inflammatory cytokines were induced by neuropathic pain [9,13]. The aim of this study was to examine the antiallodynic effects of the anti-emetic and anti-vertigo drug diphenidol in a rat neuropathic pain model. The level of tumor necrosis factor-α (TNF-α) in the sciatic nerve of chronic constriction injury (CCI) rats was assessed after diphenidol treatment.

Male adult Sprague-Dawley rats (200–250 g) from the National Laboratory Animal Center (Taipei, Taiwan) were used for the experiments. Animals with unlimited access to food and water were

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http://dx.doi.org/10.1016/j.neulet.2013.07.030

housed in a controlled environment at 22 °C with approximately 50% relative humidity on a 12-h light–dark cycle (6 a.m.–6 p.m.). The experimental protocols were approved to perform this study by the Institutional Animal Care and Use Committee of China Medical University, Taiwan. Effort was made to minimize discomfort of the animals and reduce the number of experimental animals. All studies were carefully conducted by the ethical guidelines for the usage of experimental pain in conscious animals put forth by the International Association for the Study of Pain (IASP) [30].

Diphenidol HCl was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Drug was freshly prepared in saline (0.9% NaCl) as solution before intraperitoneal injection. Animals received intraperitoneal injection of diphenidol daily and started on day 1 after CCI surgery.

Rats were randomly divided into five groups: (1) rats sham operated (SO), (2) rats with CCI combined with saline treatment (CCI), (3) rats with CCI combined with diphenidol 0.4 μmol/kg treatment (diphenidol 0.4 μmol/kg), (4) rats with CCI combined with diphenidol 2 μmol/kg treatment (diphenidol 2 μmol/kg), and (5) rats with CCI combined with diphenidol 10 μmol/kg treatment (diphenidol 10 μmol/kg). Rats were considered for the overall behavioral analysis (n = 6, 10, 10, 10, 10 for SO, CCI, diphenidol 0.4 μmol/kg, diphenidol 2 μmol/kg, diphenidol 10 μmol/kg, respectively), while some rats were killed for TNF-α analysis on day 7 after CCI (n = 5, 5, 5, 5, 5 for SO, CCI, diphenidol 0.4 μmol/kg, diphenidol 2 μmol/kg, diphenidol 10 μmol/kg, respectively). Experimenters were blind for rat assignment to different experimental groups.

To produce the CCI model, the rats were anesthetized in a bell jar with 2–3% isoflurane in 100% oxygen. Four chromic gut sutures were tied loosely around the sciatic nerve as described by Bennett and Xie [2]. The degree of constriction of the sciatic nerve was controlled by ligation and sometimes a small, brief twitch in the muscle surrounding the exposed sciatic nerve was produced [5]. Sham operation involved exposure of the sciatic nerve and its branches with the same procedures but without creating any lesion. The sciatic nerve of each rat after CCI treatment was rechecked and the integrity of the sutures was confirmed at the end of the study.

Behavioral testing was always tested between 8 and 11 a.m. at those following time points in injured or uninjured rats: 1 day before, the day of surgery, and 3, 6, 7, 11, 13, and 14 days after surgery. In addition, all animals were monitored at 0, 0.5, 1, and 2 h after diphenidol or saline administration. For consistency, an experienced investigator who was blinded to the groups was responsible for behavioral tests. For assessment of mechanical allodynia, rats were placed individually in a clear plexiglass chamber (22 cm [length] × 2 cm [width] × 13.3 cm [height]) and supported by a wire mesh floor (40 cm [width] × 50 cm [length]). An electronic von Frey filament Analgesia Meter (IITC Life Science Instruments, Woodland Hills, CA) was applied at the lateral plantar surface of rat left hind paw [5]. The paw withdrawal threshold was recorded. The withdrawal responses evoked by mechanical stimulation were determined including foot lifting, shaking, licking and squeaking, while paw movements of rats associated with locomotion or weight shifting were not counted. The mechanical stimulation was repeated 3 times at intervals of 3 min for each test and the mean was calculated.

Rats were anesthetized with urethane (1.67 g/kg, i.p.) and killed on day 7 after CCI. Under aseptic conditions, skin was cut to expose the left sciatic nerve, proximal to the trifurcation (about 1 cm), before the four ligatures were removed. The nerve specimen was immediately stored at −80 °C for the protein assay. Ice cold (4 °C) homogenization buffer was freshly prepared by adding protease inhibitor (P 8340 cocktail, Sigma–Aldrich, St. Louis, MO) to T-PER™ Tissue Protein Extraction Reagent (Pierce Chemical Co., Rockford, IL) prior to tissue lysis. After adding the buffer, a homogenization probe (Tissue Tearor, Polytron; Biospec Products, Inc., Bartlesville, OK, USA) was applied for 20 s on ice at 21,000 rpm. Then the homogenized samples were centrifuged for 40 min at a speed of 13,000 rpm at 4 °C, stored at −80 °C and used subsequently for protein quantification. The protein concentration in the supernatant was quantified using the Lowry protein assay. Samples were pipetted as duplicates (1 μl/50 μl/well) in a 96-well microtiter plate (Costar). Each plate was inserted into a plate reader (Molecular Device Spec 383, Sunnyvale, CA, USA) to read the optical density of each well at an absorbance of 750 nm. Data were analyzed using Ascent Software (London, UK) for iEMS Reader.

The concentration of TNF-α in the supernatants were determined using the DuoSet® ELISA Development Kit (R&D Systems, Minneapolis, MN). All experimental procedures were performed in accordance with the instructions. Plates were individually inserted into the plate reader for reading optical density using a 450-nm filter. Data were then analyzed using Ascent Software for iEMS Reader and a four-parameter logistics curve-fit. Data were expressed in pg/mg protein of duplicate samples.

Values in this experiment are presented as the mean ± SEM of n observations unless noted otherwise. Tests for statistical significance between multiple experimental groups was analyzed by 1-way or 2-way analysis of variance (ANOVA) with a Tukey–Kramer multiple comparison post hoc analysis. Statistical calculations were examined by SPSS for Windows (version 17.0, SPSS Inc., Chicago, IL). In each case, statistical significance was set at $P < 0.05$.

All animals were tested mechanical withdrawal threshold before treatment and 0.5, 1, and 2 h after diphenidol or saline (vehicle) administration. Regarding the similarities of those figures, only these figures obtained on day 3 or day 7 after CCI were shown (Fig. 1). On day 3 after CCI, diphenidol inhibited the CCI-induced lowering of mechanical withdrawal threshold at 1 h after the drug administration, but only when drug therapy was at 2 and 10 μmol/kg, whereas diphenidol at the dose of 0.4 μmol/kg failed to alter paw withdrawal threshold at any time point (Fig. 1A). On day 7 after CCI, diphenidol suppressed the CCI-induced lowered paw withdrawal threshold at the doses of 2 and 10 μmol/kg, whereas diphenidol at 0.4 μmol/kg did not alter significantly at any time point (Fig. 1B).

We used the data collected at 1 h after administration for Fig. 2 as diphenidol had maximal effect at 1 h after administration. Rats after CCI to the left sciatic nerve exhibited the degree of reduction (maximal 44%) from baseline in mean mechanical withdrawal threshold of the same paw before surgery (Fig. 2). In comparison with SO group, the significant difference in mechanical withdrawal threshold of CCI group was maintained from day 3 to day 14 (Fig. 2). Systemic diphenidol blocked the CCI-induced low mechanical withdrawal threshold, but only when drug therapy was at the doses of 2 and 10 μmol/kg (Fig. 2). After the day of diphenidol injection, diphenidol at 0.4 μmol/kg did not change significantly in paw withdrawal threshold at any time point throughout the experiment (Fig. 2) when compared with the CCI group. In those CCI rats that underwent diphenidol 10 μmol/kg, paw withdrawal thresholds did not significantly alter at any time point throughout the study, even 2 week after CCI (Fig. 2). Furthermore, we observed no signs of sedation and no difference in exploratory behavior and grooming at any time point during this study.

Fig. 3 depicts the expression of TNF-α in the sciatic nerve of 5 groups on day 7 after CCI. CCI rats displayed higher TNF-α expression than SO rats ($P < 0.05$). The level of TNF-α was reduced in the diphenidol 2 μmol/kg group (38 ± 16 pg/mg protein, $P < 0.05$) or diphenidol 10 μmol/kg group (6 ± 4 pg/mg protein, $P < 0.05$) compared with the CCI group (102 ± 17 pg/mg protein) on day 7 after CCI (Fig. 3). Furthermore, the expression of TNF-α is till significantly higher ($P < 0.05$) than SO even after 0.4 μmol/kg diphenidol administration (Fig. 3). Compared with CCI alone group (Fig. 3), diphenidol