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ScienceDirect
Neuroscience Research 58 (2007) 176–182
Neuroscience Research
www.elsevier.com/locate/neures

Withanoside IV improves hindlimb function by facilitating axonal growth and increase in peripheral nervous system myelin level after spinal cord injury

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Received 22 November 2006; accepted 17 February 2007
Available online 2 March 2007

Abstract

Although methylprednisolone is the clinically standard medication and almost the only therapy for spinal cord injury (SCI), its effect on functional recovery remains questionable. Transplantation strategies using sources such as neural stem cells and embryonic spinal cord still have some hurdles to overcome before practical applications become available. We therefore aimed to develop a practical medication for SCI. Per oral treatment with withanoside IV, which was previously shown to regenerate neuronal networks in the brain, improved locomotor functions in mice with SCI. In the spinal cord after SCI, axons were crushed in the white matter and gray matter, and central nervous system (CNS) myelin level decreased. In mice treated with withanoside IV (10 μmol/kg body weight/day, for 21 days), axonal density and peripheral nervous system (PNS) myelin level increased. The loss of CNS myelin and increase in reactive gliosis were not affected by withanoside IV. These results suggest that oral administration of withanoside IV may ameliorate locomotor functions by facilitating both axonal regrowth and increase in PNS myelin level.

Keywords: Spinal cord injury; Withanoside IV; Axon; PNS myelin; Hindlimb function; Mouse; Contusion

1. Introduction

Spinal cord injury (SCI) causes damage to ascending and descending fiber tracts and the loss of neurons and oligodendrocytes at and around the lesion site. Although the growth of injured axons and/or spared axons may lead to the restoration of function, axonal growth in the adult mammalian central nervous system (CNS) is inhibited in part by myelin-derived inhibitors (Schwab and Caroni, 1988; McGee and Strittmatter, 2003), and in part by glial scar-derived inhibitors (Snow et al., 1990; Bradbury et al., 2002). Several studies have suggested that the inhibition of CNS myelin inhibitory proteins may prove effective in treating SCI. For example, the blockade of Nogo-66, a myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein reportedly promotes axonal sprouting and functional recovery after SCI (Li et al., 2004). However, this strategy may not be sufficient for regenerating myelinated axon tracts under conditions of severe loss of CNS myelin. Transplantations of neural stem cells (Iwanami et al., 2005; Okada et al., 2005), embryonic spinal cord (Nakamura et al., 2005; Hase et al., 2002; Coumans et al., 2001), and bone marrow stromal cells (Neuhuber et al., 2005) have recently been actively studied as potential therapeutic methods. In practical clinical situations, however, several hurdles remain to be overcome to secure sufficient amounts of cells that are truly effective without eliciting rejection responses in patients. Methylprednisolone is used in the standard treatment of acute SCI at present, which is based on the results of clinical multi-center studies such as National Acute Spinal Cord Injury Studies II and III, and a Japanese trial (Fehlings and Baptiste, 2005). However, the effects of methylprednisolone on functional recovery have been questioned (Kronvall et al., 2005; Weaver et al., 2005). As candidates for anti-SCI drugs, several compounds have been investigated such as omega-3 fatty acids (King et al., 2006), quipazine (Brumley and Robinson, 2005), and antioxidants (Sharma et al., 2006). Although improvement of locomotion was noted in animals treated with these compounds, the extent of recovery was small. In addition, the effects of these

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0168-0102/$ – see front matter
doi:10.1016/j.neures.2007.02.014

drugs on degenerated axons and myelins were not discussed in those reports.

During the subacute phase after SCI, locomotion spontaneously, but slightly, improves (Okada et al., 2006; Leung and Wrathall, in press). At this phase, the growth of axons and the increase in the number of Schwann cells occur in the injured site (Pearse et al., 2004). Therefore, promoting the healing process after SCI must be a key strategy in treating SCI. We previously investigated the effects of extracts and constituents of Ashwagandha (root of *Withania somnifera* Dunal), an Ayurvedic tonic medicine, on neurite outgrowth in cultured neurons (Tohda et al., 2000; Zhao et al., 2002; Kuboyama et al., 2002, 2005). Withanoside IV (WS-IV) (MW: 782) was isolated from the methanol extract of Ashwagandha, and shows anti-dementia activity after its oral administration to an Alzheimer's disease mouse model (Kuboyama et al., 2006). We noted the significance of the activities of WS-IV, namely, enhancing the growth activity of neurites from injured and/or spared neurons and synaptogenesis in the brain (Kuboyama et al., 2006), and considered that these effects might also be observed following SCI. The present results indicate that oral administration of WS-IV attenuates hindlimb dysfunctions, enhances axonal regrowth, and increases peripheral myelin levels in a mouse model of contusive SCI.

2. Materials and methods

2.1. Animals and SCI model

Six-week-old ddY male mice (SLC, Shizuoka, Japan) were used in these experiments. The mice were housed with ad libitum access to food and water, and were kept under constant environmental conditions (22 ± 2 °C, 50 ± 5% humidity, 12-h light:12-h dark cycle starting at 07:00 h). Animals were handled in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Toyama, and all protocols were approved by the Animal Care Committee of the University of Toyama. Contusive injury was produced using a weight-drop model as previously described (Abe et al., 2003). T9 laminectomy was performed after anesthetization, and a 2.5-g weight was dropped three times from a height of 2 cm onto the exposed dura matter. The sham-operated group underwent laminectomy without the weight-drop. After surgery, the overlying muscles were sutured and the skin was closed with silk-braiding suture.

2.2. Drug administration

WS-IV was isolated from the methanol extract of Ashwagandha (root of *W. somnifera*, Dunal), as previously described (Zhao et al., 2002). WS-IV (1 or 10 μmol/kg body weight/day) or tap water as vehicle was administered orally once daily, from 1 h after SCI on day 0 to day 20.

2.3. Behavioral testing

Motor functions were assessed on the basis of the Basso, Beattie, Bresnahan (BBB) locomotion scale score (Basso et al., 1995) and frequency of rearing behavior. Open-field locomotion was evaluated using the 21-point BBB locomotion scale. Mice were placed in an open field (23.5 cm × 16.5 cm × 12.5 cm) and observed individually for 5 min. At the same time, the number of frequency of rearing on hindlimbs in 5 min was counted.

2.4. Immunohistochemistry

Twenty-one days after SCI, the mice were anesthetized with trichloroacetaldehyde monohydrate (chloral hydrate; Wako, Osaka, Japan) and intracardially perfused with 4% paraformaldehyde (PFA, pH 7.4). Spinal cord tissues were removed and post-fixed overnight in 4% PFA, then soaked in 10, 20 and 30% sucrose. Serial slices of spinal cords were cut as 12-μm sagittal sections using a CM3050S cryostat (Leica, Heidelberg, Germany). Slices were fixed in 4% PFA for 30 min, then immunostained. An anti-phosphorylated neurofilament-H (P-NF-H) antibody (dilution 1:1000, clone SMI35; Starnberger Monoclonals, Lutherville, MD, USA), an anti-myelin basic protein (MBP) antibody (dilution 1:100; Chemicon, Temecula, CA, USA), an anti-peripheral myelin protein-22 (PMP-22) antibody (dilution 1:500; Lab Vision, Fremont, CA, USA), and an anti-glial fibrillary acidic protein (GFAP) antibody (dilution 1:500; Chemicon) were used. Alexa Fluor 488-conjugated goat anti-mouse IgG (dilution 1:200; Molecular Probes, Eugene, OR, USA) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (dilution 1:200; Molecular Probes) were used as secondary antibodies. Slices were mounted using Aqua Poly Mount (Polysciences, Warrington, PA, USA), and viewed under an AX-80 fluorescence microscope (Olympus, Tokyo, Japan). For double immunostaining, images were captured using an LSM-GB200-IMT-2 confocal laser scanning microscope (Olympus). Fluorescence intensities of immunostained areas were measured in two to six slices per mouse using Densitograph software (ATTO, Tokyo, Japan). Areas to be measured were three squares of 50 μm × 50 μm randomly selected in the center of lesion (Fig. 2e and f), site adjacent to the center of lesion (within 300 μm from the center of lesion) (Figs. 2a–d and 4) or intact distal site (800–1100 μm apart from the center of lesion) (Figs. 2e and f and 4). To avoid inappropriate deviation of data caused by uneven staining, expression level is presented as the ratio of proximal intensity to distal intensity (Figs. 2e and f and 4). Axonal length in the white matter (220 μm × 165 μm area per slice) was measured using Neurocyte software (Kurabo, Osaka Japan). In the case of the sham-operated group, the spinal cord below the laminectomized position was isolated and immunostained. The center of a sagittal section of a sham-operated spinal cord was determined as the central position for image analyses.

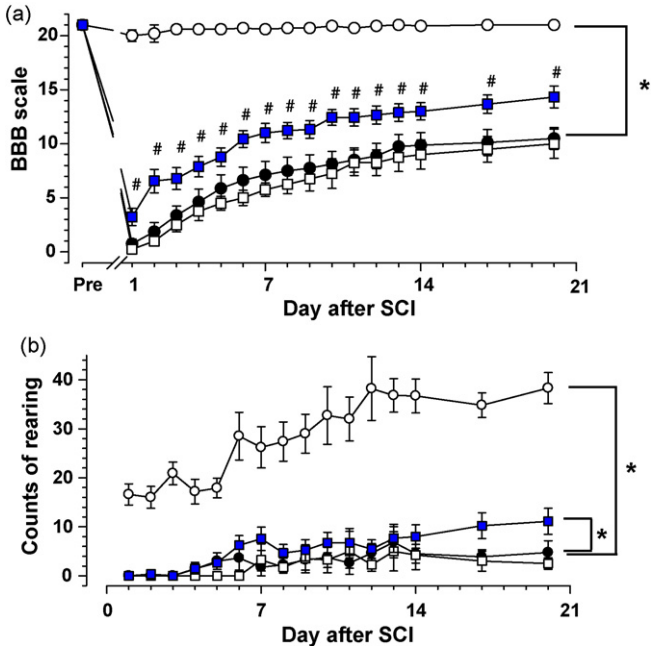

Fig. 1. Treatment with WS-IV improves hindlimb function. To the sham-operated control group, vehicle was administered (open circles, $n = 10$). To SCI mice, WS-IV at a dose of 1 μmol/kg body weight (open squares, $n = 4$) or 10 μmol/kg body weight (closed squares, $n = 9$), or vehicle alone (closed circles, $n = 8$) was administered orally for 21 days. Hindlimb behavior was assessed using a BBB locomotor scale for 20 days after SCI (a). The frequency of open-field rearing behavior on hindlimbs was determined for 5 min (b). Values represent mean and S.E.M. $^{*}P < 0.05$; $^{\#}P < 0.05$ when compared with vehicle-treated SCI group (*repeated measures two-way ANOVA; $^{\#}$Kruskal–Wallis one-way ANOVA).