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Protective role of β-carotene against oxidative stress and neuroinflammation in a rat model of spinal cord injury

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ABSTRACT

Acute spinal cord injury (SCI) results in long-lasting functional impairments through both mechanical damage as well as secondary mechanisms, with limited available therapeutic options. β-Carotene has been demonstrated to exert biological and pharmacological activities. We aimed to examine the protective effects of β-carotene in a SCI rat model. We tested the hind-limb locomotor function, neuro-inflammation, oxidative stress, astrocyte activation and nuclear factor–κB (NF-κB) pathway activation of SCI rats, with or without β-carotene treatment. β-Carotene substantially improved locomotion that was reduced by SCI. β-Carotene also relieved SCI-induced oxidative stress via regulation of reactive oxygen species, malondialdehyde, nitric oxide, and superoxide dismutase, as well as restored SCI-suppressed protein expressions of Nrf2 and HO-1. Additionally, β-carotene decreased the generation of pro-inflammatory cytokines, including tumor necrosis factor-α, interleukin-1β, interleukin-18 and cyclooxygenase-2, and inhibited the activation of astrocyte in the spinal cord. Furthermore, β-carotene treatment markedly inhibited the NF-κB pathway activation. Our findings demonstrated that β-carotene effectively reduced the progression of secondary injury events following SCI through preventing NF-κB pathway activation. Therefore, β-carotene may be an effective candidate for treating SCI.

1. Introduction

Spinal cord injury (SCI) refers to the acute traumatic insults to the neural components in the spinal canal, which induces apoptosis and death of massive neurons and oligodendrocytes, and in turn causes degeneration or demyelination of axons, leading to spinal cord motor, sensory and autonomic dysfunctions [1–4]. 130,000 individuals around the world suffer from an acute SCI annually, and there are 2,500,000 individuals living with chronic paralysis [5]. Oxidative stress and inflammation are essential players in the pathogenesis following SCI [6,7]. Glial cells (microglia and astrocytes) exert pivotal functions in the induction of inflammation, and have been suggested to contribute to the neurodegenerative process in a SCI model through releasing pro-inflammatory cytokines and reactive oxygen species (ROS) [8–10]. Astrocytes, in particular, were reported to be a fast responder to SCI, and became reactive in animal models [11]. In addition, astrocyte activation has been considered common during inflammatory after SCI [12]. The nuclear factor–κB (NF-κB) family of transcription factors (RelA/p65, c-Rel/p75, RelB/p68, p52, and p50) is critical for the expression of inflammatory genes [7]. It has also been shown that a low level of ROS activates NF-κB, which in turn modulates the transcriptional activities of the genes that encode pro-inflammatory cytokines such as interleukin-1β (IL-lβ), IL-12 and tumor necrosis factor-α (TNF-α), as well as cell adhesion molecules, inducible nitric-oxide synthase (iNOS) and cyclooxygenase (COX)-2 [13–15]. Further, inhibition of astroglial NF-κB activity was reported to stimulate corticospinal axon sparing and sprouting to promote functional recovery following SCI [16].

Diet enriched with vitamins that possess anti-oxidative capacities has attracted great research interest due to their potential health benefits against cancer, cardiovascular diseases and transplantation tissues. β-Carotene, a precursor of vitamin A, is one of naturally existing carotenoids in fruits or vegetables [17]. During the past decade, dietary supplementation with high-dose synthetic carotenoids has been successful in treating diseases associated with inflammation and oxidative stress [18,19]. In elderly population, enhanced intake of β-carotene

Abbreviations: (SCI), acute spinal cord injury; (NF-κB), nuclear factor–κB; (ROS), reactive oxygen species; (MDA), malondialdehyde; (NO), nitric oxide; (SOD), superoxide dismutase; (TNF-α), tumor necrosis factor-alpha; (IL-1β), interleukin-1 beta; (IL-18), interleukin-18

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reduces body fat and triglyceride, thereby alleviating metabolic syndromes. Further, a high proportion of vegetables and fruits in the diet sustains healthy plasma carotenoid levels and reduces the risk of ischemic stroke [19]. β-Carotene has also been shown to be a potent inhibitor of oxidative stress and inflammation in vitro and in vivo. Treatment of β-carotene effectively suppressed the expression of pro-inflammatory cytokines, such as IL-1β and IL-6, as well as oxidants induced by ischemia injury [20]. In human umbilical vein endothelial cells, β-carotene and lycopene both greatly affected TNF-α-induced inflammation via reducing productions of ROS, nitrotyrosine and NF-κB [21]. Recent investigations have reported that β-carotene regulates the DNA binding activity of NF-κB in human colon adenocarcinoma and leukemia cells [22]. β-Carotene also suppressed the inflammatory gene expressions by inhibiting NF-κB activity in lipopolysaccharide-stimulated macrophages [23]. These evidences suggest that β-carotene may be an anti-oxidant by inhibiting the NF-κB pathway. Thus, we hypothesized that β-carotene might play protective roles against SCI by suppressing the NF-κB signaling pathway.

The current investigation aimed to study the functions of β-carotene in a rat SCI model. We assessed the effects of β-carotene on the post-traumatic oxidative stress and inflammatory responses following SCI. We also investigated the potential involvement of NF-κB pathway for a better insight into the mechanisms underlying β-carotene actions.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (body weight 250–300 g) were provided by Animal Center in Shanghai Jiaotong University. Animals were housed in a controlled environment (22 ± 1 °C with a 12:12 h light/dark cycle) with free access to water and food. The surgical and animal care procedures were in compliance with the guidelines of the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments were carried out in conformity with Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

2.2. SCI model

A total of 96 rats were used in the current study including 48 rats for hind-limb locomotor evaluation and the other 48 rats for biochemical and histological analyses. Animals were anaesthetized using chloral hydrate (400 mg/kg, Sigma, St. Louis, MO). Additional 203 rats were used for the biologically repeated experiments. The SCI model was established as described previously with some modifications [24]. Briefly, a longitudinal cut was made on the back along the midline, and the paravertebral muscles were surgically collected. The spinal cord from T5 to T8 was exposed via laminectomy. At the T6 to T7 level, the spinal cord was compressed in the bilateral direction using an aneurysm clip with a closing force of 70 g for 1 min to build the spinal cord injury model. Animals in the sham-operated group were subjected only to laminectomy. Following SCI, the bladders of the rats were voided manually twice a day until normal functions of bladder were restored. To study the protective effects of β-carotene on SCI, rats received 10, 20, 40, and 80 mg/kg β-carotene intraperitoneally once immediately after the surgery, with saline as vehicle. Spinal cord tissue was dissected and collected at 72 h after trauma.

2.3. Hind-limb locomotor evaluation

The Basso, Beattie and Bresnahan (BBB) score test was used to determine the extent of hind-limb locomotor deficits after SCI. The score range in BBB test is from 0 for complete paraplegia to 21 for normal locomotion [25]. To evaluate the changes in locomotion after SCI, rats were trained twice a week before the surgery to explore freely for 4 min in an open field apparatus, a circular enclosure with a smooth nonslip floor, and their abilities to use hind-limbs were recorded. Joint movements, weight support, paw placement and fore/hind-limb coordination were scored using the 21-point BBB locomotion scale. 24 h post operation hind-limb motor function was assessed again. All behavioral experiments were conducted by two observers blind to the group assignments.

2.4. Histopathological evaluation (HE) staining

Rats were fixed with 4% paraformaldehyde, and the spinal cord samples were collected 72 h post SCI. The samples were embedded in paraffin after dehydration with ethanol at gradient concentrations. Coronal sections at 5 μm thickness were cut and underwent hematoxylin and eosin staining. A light microscope (Olympus BH-2; Olympus Corp., Tokyo, Japan) was used to assess sections. Counting of damaged neurons was performed and the histopathological alteration of the gray matter was scored on a 6-point scale: 0 = no observable lesion; 1 = gray matter contained 5–10 eosinophilic neurons; 3 = gray matter contained > 10 eosinophilic neurons; 4 = < 1/3 of the gray matter area infarction; 5 = 1/3–1/2 of the gray matter area infarction; 6 = > 1/2 of the gray matter area infarction [5]. The pathological score for an individual animal was calculated as the average of all the sections from one spinal cord. All the histological examination was conducted in a blind manner.

2.5. Biochemical analyses

2.5.1. Tissue melondialdehyde (MDA) and superoxide dismutase (SOD) levels

MDA level was evaluated using a thiobarbituric acid reaction-based method as previously described [25]. In brief, homogenized sample (100 μl) was added to 50 μl sodium dodecyl sulfate (8.1%), then mixed by vortexing, followed by incubation at room temperature for 10 mins. Thiobarbituric acid (0.6%) and 20% acetic acid (pH 3.5) at equal volumes were then added and heated in boiling water for 1 h. 1.25 ml butanol-pyridine mixture (15:1) was added to each sample after the samples cooled down to room temperature. The samples were vortexed and then centrifuged at 12000*g* for 10 min. The color of the supernatant was measured at 532 nm. The concentration of MDA was calculated as nmol/mg protein.

Total SOD activity was measured using previously established method [26]. SOD-induced suppression of nitroblue tetrazolium reduction was measured with the use of a xanthine–xanthine oxidase system, which served as a superoxide generator. One milliter ethanol-chloroform mixture (in volume 5:3) was added to 1 ml tissue homogenate and subjected to centrifugation. Soon afterwards, activities were evaluated in the ethanol phase of the supernatant. One unit of SOD activity was defined as the amount of enzyme that caused a 50% suppression of nitroblue tetrazolium reduction. Activities of SOD were quantified as unit/mg protein.

2.5.2. Tissue ROS and nitric oxide (NO) levels

ROS production in the spinal cord (n = 5 for each group) was measured 72 h following SCI using the oxidative fluorescent dye dihydroethidium (DHE). Spinal cord cryosections (10 μm thickness) were incubated with DHE for half an hour, and then equilibrated with phosphate-buffered saline (PBS) at 37 °C for another 30 min. Oxidized DHE was assessed using fluorescence microscopy (TE; Nikon, Tokyo, Japan).

NO level was measured as described previously [27]. The spinal cord samples homogenized in 10 ml ice-cold saline solution were mixed with absolute ethanol for protein precipitation. Following 15 min separation at 25 °C, the supernatant was collected and mixed with equal volumes of vanadium (III) chloride, and 0.5 ml freshly prepared Griess reagent (0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride, 2% phosphoric acid and 1% sulfanilamide) was immediately added. The