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Lycopene ameliorates neuropathic pain by upregulating spinal astrocytic connexin 43 expression

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ABSTRACT

Aim: Peripheral nerve injury upregulates tumor necrosis factor (TNF) expression. In turn, connexin 43 (Cx43) expression in spinal astrocytes is downregulated by TNF. Therefore, restoration of spinal astrocyte Cx43 expression to normal level could lead to the reduction of nerve injury-induced pain. While the non-provitaminic carotenoid lycopene reverses thermal hyperalgesia in mice with painful diabetic neuropathy, the antinociceptive mechanism is not entirely clear. The current study evaluated whether the antinociceptive effect of lycopene is mediated through the modulation of Cx43 expression in spinal astrocytes.
Main methods: The effect of lycopene on Cx43 expression was examined in cultured rat spinal astrocytes. The effect of intrathecal lycopene on Cx43 expression and neuropathic pain were evaluated in mice with partial sciatic nerve ligation (PSNL).
Key findings: Treatment of cultured rat spinal astrocytes with lycopene reversed TNF-induced downregulation of Cx43 protein expression through a transcription-independent mechanism. By contrast, treatment of cultured spinal astrocytes with either pro-vitamin A carotenoid β-carotene or antioxidant *N*-acetyl cysteine had no effect on TNF-induced downregulation of Cx43 protein expression. In addition, repeated, but not single, intrathecal treatment with lycopene of mice with a partial sciatic nerve ligation significantly prevented not only the downregulation of Cx43 expression in spinal dorsal horn but mechanical hypersensitivity as well.
Significance: The current findings suggest a significant spinal mechanism that mediates the analgesic effect of lycopene, through the restoration of normal spinal Cx43 expression.
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1. Introduction

Neuropathic pain arises as a symptom from a number of diseases or exposure to toxic chemicals and is often chronic and refractory to treatment with standard analgesics such as opioids and non-steroidal anti-inflammatory drugs. Depending on the etiology, a number of distinct mechanisms induce and maintain the neuropathic pain state. Thus, “neuropathic pains” are differentially sensitive to pharmacological treatments, which hampers truly effective management of neuropathic pain in general [1].

Connexin43 (Cx43) is mainly expressed in CNS astrocytes, and has a pivotal role in the formation of gap junctions [2]. Gap junctions facilitate intercellular communication by passing signaling molecules such as glutamate, ATP, and second messengers, buffering extracellular Na^+ and K^+, and supplying energy sources between neighboring cells [3–6]. Gap junctions are formed by two connexons, which, in turn, consists of a hexamer of connexins protein. Spinal astrocytic Cx43 also have essential roles in nociceptive transduction in the neuropathic pain state [7]. More specifically, astrocytic Cx43 underlies the maintenance, rather than the induction, of neuropathic pain. While mechanical hypersensitivity is observed 3 days after partial sciatic nerve ligation (PSNL), downregulation of astrocytic Cx43 in the ipsilateral lumbar spinal dorsal horn is observed beginning 7 days nerve ligation [8]. Furthermore, restoration of normal Cx43 levels by an adenovirus vector expressing Cx43 is accompanied by amelioration of PSNL-induced mechanical hypersensitivity [8]. This result suggests that reversing Cx43 downregulation could be a potent therapeutic approach for the treatment of neuropathic pain.

Various compounds can affect the regulation of Cx43 expression. Among these is lycopene, a non-provitaminic carotenoid, found in abundance in tomatoes and in products utilizing tomatoes. Lycopene appears to have significant benefits in reducing the risk of developing chronic diseases such as cancer and cardiovascular disease [9,10]. The

Abbreviations: Cx43, connexin43; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; NAC, *N*-acetyl cysteine; PSNL, partial sciatic nerve ligation; RAR, retinoic acid receptor; RARE, retinoic acid response element; TNF, tumor necrosis factor.

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benefits of lycopene could be attributed to not only its antioxidative action but also its capacity to upregulate Cx43 expression in cancer cells [11,12]. The over-expression of Cx43 leads to decreased proliferation in melanoma cells [13].

Systemic lycopene reduced serum levels of nitric oxide and tumor necrosis factor (TNF) and ameliorated thermal hyperalgesia in a mouse model of painful diabetic neuropathy, which suggests that lycopene reduces neuropathic pain through an anti-inflammatory mechanism [14]. Alternatively, TNF has been shown to directly downregulate Cx43 expression in astrocytes both in vitro and in vivo [8,15]. Therefore, it is possible that lycopene could reduce neuropathic pain by modulating the expression of Cx43 in spinal astrocytes in addition to an anti-inflammatory mechanism. In the current study, a potential modulatory role of lycopene on Cx43 expression in a model of a painful peripheral neuropathy was examined. To determine whether or not lycopene directly modulates Cx43 expression, specifically in astrocytes, Cx43 expression in cultured spinal astrocytes treated with TNF was examined. In addition, to confirm that lycopene's in vitro effect on Cx43 expression in spinal astrocyte leads to significant in vivo analgesia, lycopene was intrathecally administered in mice with a PSNL.

2. Materials and methods

2.1. Animals

Male ddy mice, 5 weeks of age, were used (Kyudo, Co., Ltd., Tosu, Japan). Mice were maintained in a vivarium, room temperature of 22 ± 2 °C, with a 12 h light/dark cycle (lights on/off at 8:00 AM/8:00 PM), and given access to food and water available ad libitum during the experimental period. All experiments utilizing animals were conducted in accordance with the “Guidelines for the Care and Use of Laboratory Animals” established by The Japanese Pharmacological Society and Hiroshima University, and procedures were reviewed and approved by the Committee of Research Facilities for Laboratory Animal Science of Hiroshima University.

2.2. Mouse partial sciatic nerve ligation (PSNL) model of neuropathic pain

Under sodium pentobarbital (50 mg/kg, i.p.) anesthesia, a tight ligation of approximately one-third to one-half of the diameter of the left sciatic nerve (ipsilateral) was performed with 8-0 silk suture as described previously [8,16]. In sham-operated mice, the sciatic nerve was exposed but not ligation. Mice with a PSNL that did not show robust mechanical hypersensitivity (hind paw withdrawal threshold >0.1 g) were excluded from the experiments. The percentage of mice that demonstrate a withdrawal threshold of >0.1 g following PSNL surgery is about 5%.

2.3. Mouse intrathecal injection

Intrathecal injections were performed on unanesthetized mice [16,17]. In brief, the vertebral landmarks for L5 and L6 vertebrate were identified by palpation. An injection into the subarachnoid space between the L5 and the L6 vertebrae was done via a 27-gage needle. Entry of the needle was confirmed with the presence of a tail flick. Lycopene (10 nmol/5 μl) was intrathecally injected either once or four times (see Section 2.4). Lycopene was dissolved in 30% DMSO.

2.4. Hind paw sensitivity to mechanical stimulation and testing schedule

The withdrawal threshold (in grams) of the hind paw to mechanical stimulation was determined using von Frey filaments [8]. In brief, the von Frey filament was pressed against the mid-planter surface of the hind paw. The lowest force that caused responses such as lifting and licking of the hind paw was assigned as the withdrawal threshold. Each hind paw was tested three times, at 10 second intervals, and the mean withdrawal threshold was reported. All behavioral tests were performed blinded.

In mice with either a PSNL or sham surgery, single treatment with lycopene or vehicle (30% DMSO in saline) was performed 14 days following surgery. Following intrathecal injection, withdrawal thresholds were measured 0.5, 1, 2, 3 and 24 h post-injection. Repeated intrathecal administration of either lycopene or vehicle was performed 7, 9, 11 and 13 days following PSNL (Fig. 4a). Withdrawal thresholds were measured 24 h after the last intrathecal injection, i.e., 14 days following PSNL. After measurement of withdrawal thresholds, Cx43 expression in the spinal dorsal horn was assessed with Western blotting. Under ether anesthesia, mice were decapitated and the lumbar (L4–L6) segments of the ipsilateral side of the spinal dorsal horn were removed. These were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.5. Materials

Lycopene was purchased from WAKO Pure Chemical Industries (Osaka, Japan). β-carotene was obtained from Cayman Chemical (Ann Arbor, MI, USA). *N*-acetyl cysteine (NAC) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lycopene and β-carotene were dissolved in 30% DMSO. NAC was dissolved in saline. Recombinant rat tumor necrosis factor (TNF) was obtained from Wako Pure Chemicals Industries (Osaka, Japan).

2.6. Cell culture

Stimulation of cultured spinal astrocytes with TNF (10 ng/ml for 48 h) significantly reduces the expression of Cx43 [15,18]. In addition to lycopene, other carotenoids can induce Cx43 expression as well [19]. Thus, in addition to lycopene (20 μM), the effect of the provitamin A carotenoid β-carotene (10, 20 μM) and the potent antioxidant NAC (0.5, 1 mM) on the TNF-induced downregulation of Cx43 expression in cultured spinal astrocytes was examined.

The preparation of cultured spinal astrocytes has been described previously [20,21]. In brief, spinal cords isolated from neonatal Wistar rats (Japan SLC, Inc., Shizuoka, Japan) were minced, and then incubated with trypsin and DNase I. Dissociated cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively). Thereafter, cell suspensions were plated in $75 cm^2$ tissue culture flasks ($7.5–10 \times 10^6$ cells/flask) precoated with poly-L-lysine (10 μg/ml). Cells were maintained in a 10% CO_2 incubator at 37 °C. After 10 days, the flasks containing mixed glial cells were vigorously shaken and washed with PBS to remove microglial cells. Remaining cells were trypsinized, and seeded to new flasks. After 7 days of incubation, the flasks were again vigorously shaken and washed, and the cells were trypsinized. Thereafter, the remaining cells were transferred to 35-mm dishes ($3–3.5 \times 10^5$ cells). After 3 days, the medium was replaced with DMEM without FCS and antibiotics. After an additional 24 h of incubation, the cells were used in experiments. Most if not all of the cells obtained using the current method were astrocytes as confirmed by RT-PCR and Western blotting [21].

2.7. Western blot

Cultured spinal astrocytes and spinal tissues were solubilized in radioimmunoprecipitation assay buffer with inhibitors (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and phosphatase inhibitor cocktail 2 (Nacalai Tesque, Kyoto, Japan)). The lysates were centrifuged at 14,000 ×g for 10 min at 4 °C and the supernatant was added to Laemli's buffer and boiled for 5 min. Equal amounts of protein were separated by 7.5 or 10% SDS-polyacrylamide gel

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