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Original Article

Xanthotoxol attenuates neuropathic pain in a rat model of chronic constriction injury

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Abstract: Xanthohumol (XN) is a prenylated chalcone derived from hops (*Humuluslupulus* L.). Several studies showed that XN exhibited potent neuroprotective activity. However, the role of XN in neuropathic pain is still unclear. In the present study, we investigated the effects of XN on neuropathic paininduced by rat sciatic nerve chronic constriction injury (CCI). The current study demonstrated that XN significantly reversed CCI-decreased thermal withdrawal latency and mechanical with drawal threshold; XN also obviously inhibited CCI-induced the levels of pro-inflammatory cytokines TNF-α and IL-1β in spinal cord. Furthermore, XN reduced the elevated expression of phospho-ERK and NF-κB p65 in spinal cord induced by CCI. Taken together, XN may have reduced neuropathic pain by mechanisms related to inhibition of the activation of ERK and NF-κB signaling pathways. Therefore, the present study suggests the potential use of XN in the treatment of neuropathic pain.

Keywords: Xanthohumol (XN), neuropathic pain, chronic constriction injury (CCI)

Introduction

Neuropathic pain is characterized by allodynia, hyperalgesia and spontaneous pain [1]. It has become a notable public health problem that affects a broader population worldwide [2]. The typical treatments for neuropathic pain are anti-depressant, anti-convulsant, or topical agents [3, 4]; however, there are still no satisfactorily effective treatments [5]. The main obstacle hampering the development of effective therapeutics is that the molecular mechanism underlying neuropathic pain remains poorly understood. Therefore, there is still a considerable need to explore novel treatment modalities for neuropathic pain management.

Xanthohumol (XN) is a prenylated chalcone derived from hops (*Humuluslupulus* L.). A growing body of evidence demonstrated that XN suppressed cancer cell proliferation and invasion*in vitro* in several solid tumors such as breast, colon, hepatocellular, medullary thyroid, ovarian, pancreatic, and prostate [6-8]. In addition to its promising anti-tumorigenic ability, XN could inhibit inflammatory cytokine production in lipopolysaccharide (LPS)-activated human monocytic THP-1 cells [9]. Most notably, XN exhibited potent neuroprotective activity, treatment with XN dose-dependently attenuated focal cerebral ischemia and improved neurobehavioral deficits in cerebral ischemic rats [10]; XN also significantly inhibited the excessive production of inflammatory mediators NO, IL-1β, and TNF-α, and the activation of NF-κB signaling in LPS-induced stimulated BV2 cells [11]. However, the role of XN in neuropathic pain is still unclear. In the present study, we investigated the effects of XN on neuropathic paininduced by rat sciatic nerve chronic constriction injury (CCI).

Materials and methods

Animals

Male Sprague-Dawley rats weighing 180-200 g were supplied by the Experimental Animal Centre of the First Affiliated Hospital of Wenzhou Medical University (China). Animals were maintained on a 12-hlight/dark cycle (lights on at 8:00 a.m., lights off at 8:00 p.m.) under controlled temperature (22±1°C), and were given a standard diet and water ad libitum. Animal

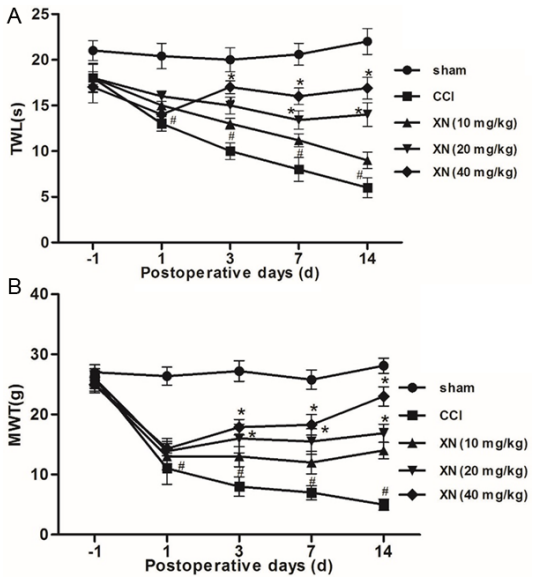

Figure 1. Effects of xanthotoxol (XN) on thermal hyperalgesia and mechanical allodynia.The thermal withdraw latency TWL (A) and mechanical withdraw threshold MWT (B) were measured after intraperitoneal (i.p.) injection of various XN (10, 20 and 40 mg/kg). Data are shown as mean ± SD. N=3, #P<0.05 vs. sham group; *P<0.05 vs. CCI group.

experiments conformed to the guidelines issued by the First Affiliated Hospital of Wenzhou Medical University. The present study was performed with approval from the Animal Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

Drug treatment

XN was obtained from Sigma (St. Louis, MO, USA). XN in doses of 10, 20 and 40 mg/kg was administered intraperitoneally (i.p.) to neuropathic rats once a day for 10 days, starting from the first day after surgery; the sham-operated mice received vehicle alone following the same treatment procedure.

Induction of neuropathic pain

A rat model of neuropathic pain was induced using a method described previously [12]. In brief, rats were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (40 mg/kg; Merck, Darmstadt, Germany). The left sciatic nerve was exposed and ligated with 4-0 catgut thread (Johnson & Johnson, New Brunswick, NJ, USA) at 4 sites with an interval of 1 mm. Sham-operated rats were performed with left sciatic nerve exposure, but without ligation.

Evaluation of thermal hyperalgesia and mechanical allodynia

Thermal hyperalgesia was tested using Hargreaves' method [13]. In brief, the rats were placed in smaller clear plexiglass cubicles and allowed to acclimatise before a constant-intensity radiant heat source (beam diameter 0.5 cm; intensity 20 IR) was aimed at the mid-plantar area of the hind paw, and the time in seconds (s) between the activation of the heat source and paw withdrawal was recorded.

The mechanical hyperalgesia was assessed by the pinprick test asdescribed previously [14]. The surface of the injured hind paw was touched with the point of the bent gauge needle (at 90° to the syringe) at intensity sufficient to produce a reflex withdrawal response. The paw withdrawal duration was recorded inseconds and the normal quick reflex withdrawal response was giventhe value of 0.5 s.

Western blot

Proteins were extracted from spinal cord tissues using cell lysis buffer and the protein concentration was quantified by the Bradford assay. Proteins were separated on SDS-PAGE containing 10% acrylamide gels and then transferred to PVDF membranes. The membranes were then incubated with a 1:1000 dilution of the following antibodies: phospho-ERK, ERK, and NF-κB p65 (all from Santa Cruz Bio technology Inc., Santa Cruz, CA, USA). Then, the blots were washed and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After being washed again with TBST buffer for 10 min, proteins were detected using enhanced chemiluminescence (Pierce, Rockford, USA).

Enzyme linked immunosorbent assay (ELISA)

Rats were anesthetized with sodium pentobarbital 24 h after the last treatment. Lumbar segments of the spinal cord were removed and homogenized in lysis buffer. After centrifugation, the supernatants were measured using commercially available rat TNF-α and IL-1β ELISA kits (Cell Biolabs, USA) according to the manufacturer's instructions.

Statistical analysis

All data were presented as the mean ± SD. The data of behavioral tests were analyzed using

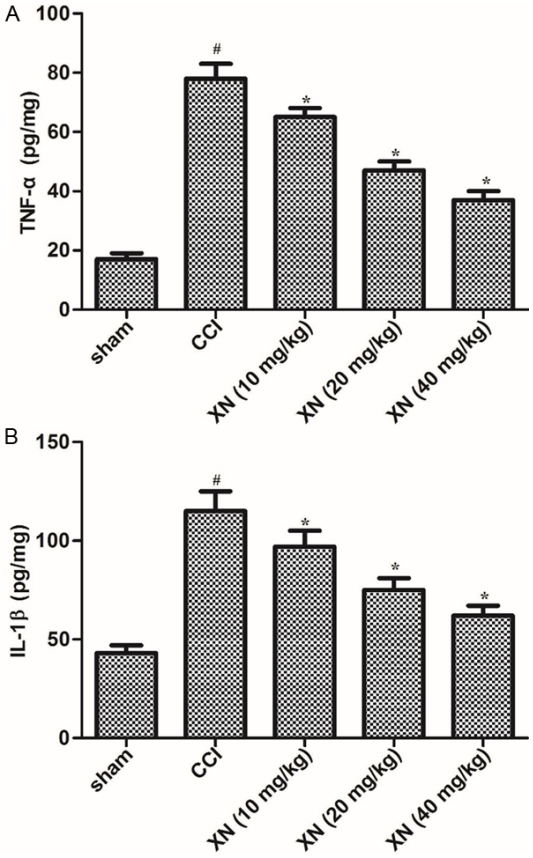

Figure 2. Effects of xanthotoxol on pro-inflammatory cytokines in spinal cord after CCI. The rats were sacrificed 24 h after the last administration. The expression of TNF-α (A) and IL-1β (B) protein was detected by ELISA. Data are shown as mean ± SD. N=3, #P<0.05 vs. sham group; *P<0.05 vs. CCI group.

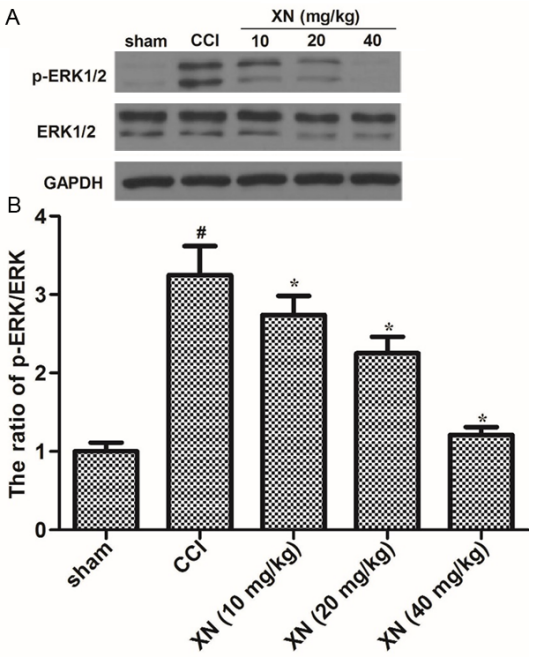

Figure 3. Effects of xanthotoxol on ERK activation in spinal cord after CCI. The rats were sacrificed 24 h after the last administration. (A) The proteins were extracted from spinal cord tissues using RIPA lysis buffer. (A) The protein level of phospho-ERK in spinal cord was determined by western blot, GAPDH was used as a loading control. Quantification analysis of relative protein levels of phospho-ERK (B) was performed using Gel-Pro Analyzer version 4.0 software. Data are shown as mean ± SD. N=3, #P<0.05 vs. sham group; *P<0.05 vs. CCI group.

two way ANOVA, while the data of cytokine assay were analyzed using one way ANOVA followed by Bonferonni'spost test. Values of $P<0.05$ were considered as statistically significant.

Results

Effects of xanthotoxol on thermal heperalgesia and mechanical allodynia

Thermal withdrawal latency (TWL) and mechanical withdrawal threshold (MWT) were assessed before surgery and on days 1, 3, 7, and 14 after CCI. As shown in **Figure 1**, CCI resulted in significant development of thermal hyperalgesia and mechanical allodynia as compared to sham group as assessed on day 1st, 7th and 14th, respectively. However, administration of XN (10, 20 and 40 mg/kg) significantly attenuated CCI-induced thermal hyperalgesia and mechanical allodynia in a dose dependent manner.

Effects of xanthotoxolon pro-inflammatory cytokines in spinal cord after CCI

Previous studies have shown that inflammatory cytokinesare involved in the modulation of neuropathic pain. So, we investigated the effects of XN on pro-inflammatory cytokines in spinal cord. As shown in **Figure 2**, CCI resulted in significant elevation of TNF-α and IL-1β in spinal cord on the 14th day as compared to sham control rats. However, administration of XN obviously attenuated CCI-induced the production of TNF-α and IL-1β.

Effects of xanthotoxol on ERK activation in spinal cord after CCI

Previous studies demonstrated that the activation of ERK in the nociceptive pathway contributes to the development of inflammatory and nerve injury induced neuropathic pain. Therefore, we investigated the effects of XN on phosphorylation of ERK in spinal cord. As shown in

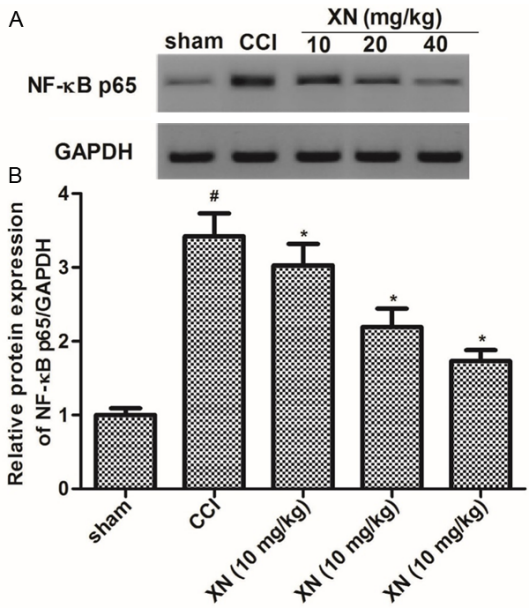

Figure 4. Effects of xanthotoxol on NF-κB activation in spinal cord after CCI. The rats were sacrificed 24 h after the last administration. The proteins were extracted from spinal cord tissues using RIPA lysis buffer. (A) The protein level of NF-κB p65 in spinal cord was determined by western blot, GAPDH was used as a loading control. Quantification analysis of relative protein levels of NF-κB p65 (B) was performed using Gel-Pro Analyzer version 4.0 software. Data are shown as mean ± SD. N=3, #P<0.05 vs. sham group; *P<0.05 vs. CCI group.

Figure 3, phospho-ERK expression up-regulated significantly in CCI-induced spinal cord tissues compare with sham control group. On the other hand, treatment of XN dramatically down-regulated phospho-ERK expression.

Effects of xanthotoxol on NF-κB activation in spinal cord after CCI

It has been reported that NF-κB may be involved in the modulation of neuropathic pain. Thus, we investigated the effect of XN on NF-κB activation in spinal cord of CCI rats. As shown in **Figure 4**, compared with the sham group, CCI significantly increased the expression of NF-κB p65. Whereas, administration of XN markedly decreased the expression of NF-κB p65 in spinal cord of CCI rats.

Discussion

The current study demonstrated that XN significantly reversed CCI-decreased thermal withdrawal latency and mechanical withdrawal threshold; XN inhibited CCI-induced the levels of pro-inflammatory cytokines TNF-α and IL-1β in spinal cord. Furthermore, XN reduced the elevated expression of phospho-ERK and NF-κB p65 in spinal cord induced by CCI.

Inflammatory mediators have diverse mechanisms and sites of action, including the activation and sensitization of nociceptive terminals [15]. The pro-inflammatory activities of TNF-α and IL-6 are well known, and these inflammatory cytokines play an important role in mediating neuropathic pain [16, 17]. Previous studies demonstrated that anti-TNF-α treatment resulted in a significant alleviation of pain in a rat CCI model [18]. Intrathecal application of IL-1β induce mechanical allodynia [19], and IL-1 receptor antagonist can inhibit hyperalgesic responses to LPS, IL-1β, carrageenan, bradykinin and TNF-α [20]. Additionally, XN was found to inhibit production of pro-inflammatory cytokines, including TNF-α and monocyte chemoattractant protein-1 (MCP-1) in LPS-activated RAW 264.7 mouse macrophages and U937 human monocytes [21]. Herein, we found that XN significantly suppressed CCI-induced the production of TNF-α and IL-1β in spinal cord of CCI rats. These data suggest that the analgesic effect XN exhibited anti-analgesic effect through the inhibition of the production of TNF-α and IL-1β.

Numerous studies have found that the inflammatory responses are linked with an increase in transcription of inflammatory genes by transcription factors capable of modulating the expression of a wide range of genes involved in neuropathic pain [22-24]. It has been demonstrated that ERK plays a critical role in pain processing by regulating peripheral and spinal neuronal sensitization initiated by neuropathic stimuli [25-27]. The activation of ERK contributes to hyperalgesia by increasing the production of cytokines and other mediators and their receptors. Activated ERK translocates from the cytosol into the nucleus and in turn phosphorylates cAMP response element binding protein (CREB) [28], and intrathecal injection of CREB antisense oligonucleotide attenuatestactile allodynia caused by partial sciatic nerve ligation [29]. Herein, we observed that treatment of XN inhibited CCI-induced phospho-ERK expression in spinal cord tissues.

Besides the ERK pathway, NF-κB also has been implicated in the mediation of neuropathic pain [30-32]. NF-κB mediates immune and inflammatory responses via the regulation of genes that can encode pro-inflammatory cytokines, adhesion molecules, and chemokines in the spinal cords after peripheral nerve injury [33, 34]. Intrathecal infusion of the NF-κB inhibitor (PDTC) improved mechanical allodynia and down-regulated the over-expression of TNF-α and tumor necrosis factor receptor 1 (TNFR1) induced by peri-sciatic administration of TNF [34]. Herein, we found that administration of XN markedly decreased the expression of NF-κB p65 in spinal cord of CCI rats. All these data suggest that XN could attenuate neuropathic pain in CCI rats by inhibiting the activation of ERK and NF-κB signaling pathways.

To conclude, XN may have reduced neuropathic pain by mechanisms related to inhibition of the activation of ERK and NF-κB signaling pathways. Therefore, the present study suggests the potential use of XN in the treatment of neuropathic pain, which merits further clinical investigation.

Disclosure of conflict of interest

None.

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