Urotensin II inhibitor eases neuropathic pain by suppressing the JNK/NF- κ B pathway

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Abstract

Urotensin II (U-II), a cyclic peptide originally isolated from the caudal neurosecretory system of fishes, can produce proinflammatory effects through its specific G protein-coupled receptor, GPR14. Neuropathic pain, a devastating disease, is related to excessive inflammation in the spinal dorsal horn. However, the relationship between U-II and neuropathic pain has not been reported. This study was designed to investigate the effect of U-II antagonist on neuropathic pain and to understand the associated mechanisms. We reported that U-II and its receptor GPR14 were persistently upregulated and activated in the dorsal horn of L4–6 spinal cord segments after chronic constriction injury (CCI) in rats. Intrathecal injection of SB657510, a specific antagonist against U-II, reversed CCI-induced thermal hyperalgesia and mechanical allodynia. Furthermore, we found that SB657510 reduced the expression of phosphorylated c-Jun N-terminal kinase (p-JNK) and nuclear factor- κ B (NF- κ B) p65 as well as subsequent secretion of interleukin-1 β (IL-1 β), IL-6 and tumor necrosis factor- α (TNF- α). It was also showed that both the JNK inhibitor SP600125 and the NF- κ B inhibitor PDTC significantly attenuated thermal hyperalgesia and mechanical allodynia in CCI rats. Our present research showed that U-II receptor antagonist alleviated neuropathic pain possibly through the suppression of the JNK/NF- κ B pathway in CCI rats, which will contribute to the better understanding of function of U-II and pathogenesis of neuropathic pain.

Key Words

- Urotensin II antagonists
- neuropathic pain
- JNK
- NF- κ B

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Introduction

Neuropathic pain can be defined as an abnormal pain sensation in the peripheral or central nervous system after injuries. It is caused by dysfunction in the peripheral or central nervous system without peripheral nociceptor stimulation (Ro & Chang 2005). Although we incompletely understand the mechanisms for development and maintenance of neuropathic

pain, it has been recognized that neuropathic pain is caused by central and peripheral neuron sensitization induced by the sustained inflammatory reaction in spinal dorsal horn, characterized by the generation of all kinds of proinflammatory cytokines such as IL-1 β , IL-6 and TNF- α (Gustafson-Vickers *et al.* 2008, Gao *et al.* 2009).

Urotensin II (U-II) was discovered from a goby fish indigenous to the Californian coastline in the 1960s. The distribution of U-II is not limited to the fish, as the cDNA encoding the pre-pro-U-II has been characterized in frog, mouse, rat and human (Dun *et al.* 2001). In 1999, Ames *et al.* (1999) found that GPR14, a kind of orphan G protein-coupled receptor, is the only high-affinity receptor of U-II. U-II and its special receptor, GPR14, are widely distributed in peripheral and central organs. With further research, it has been proven that U-II combined with GPR14 can produce a variety of biological effects, especially its proinflammatory effects. Many studies have reported that U-II exerts potent proinflammatory effects in the metabolic syndrome (Barrette & Schwertani 2012), cardiovascular disease (Hassan *et al.* 2005), hepatic disease (Liang *et al.* 2014) and so on (Johns *et al.* 2004, Tabur *et al.* 2015), followed by the generation of a variety of proinflammatory cytokines including IL-1 β , IL-6 and TNF- α . It was reported that U-II can significantly stimulate the phosphorylation of JNK (c-Jun N-terminal kinase) and enhance the expression of c-fos mRNA in rat cardiomyocytes (Chiu *et al.* 2014). In addition, the expression of U-II can be upregulated by the inflammatory cytokines. ROS can significantly promote the expression of U-II (Shyu *et al.* 2012). IL-1 β , IL-6 and TNF- α markedly increased the expression of U-II through the ERK1/2 (extracellular signal-regulated kinase1/2) and NF- κ B (nuclear factor kappa B) pathway in human peripheral blood mononuclear cells (Segain *et al.* 2007). Recent studies have identified that U-II and GPR14 are also distributed in the central nervous system (Ames *et al.* 1999, Coulouarn *et al.* 1999, Liu *et al.* 1999, Dun *et al.* 2001) including the spinal neurons and glial cells (Takahashi *et al.* 2001, Lin *et al.* 2003, Egginger *et al.* 2006). However, the roles of U-II and its receptor GPR14 in the spinal cord remain to be clarified.

The neuron sensitization induced by sustained inflammatory reaction in spinal dorsal horn plays important roles in the development and maintenance of neuropathic pain. Moreover, U-II can exaggerate the inflammatory reaction by interacting with proinflammatory cytokines IL-1 β , IL-6 and TNF- α . We, therefore, hypothesized that U-II has the ability to modulate the development of neuropathic pain. In the present study, we first investigated the expression changes of U-II and its receptor GPR14 in the spinal dorsal horn of the neuropathic pain rats induced by chronic constriction injury (CCI). Second, we evaluated the effect of U-II receptor antagonist SB-657510 on the

development of neuropathic pain and further explored the relevant mechanisms.

Materials and methods

Animals

Male Sprague–Dawley (SD) rats weighing 200–220 g were used. Animals were housed in private cages at a temperature of 20°C with a 12-h light-darkness cycle (light on 08:30–20:30) and fed with food and water *ad libitum*. The animal experiments were approved by the Animal Ethics Committee of Xuzhou Medical University and were in agreement with the official recommendations of the Chinese Community Guidelines (XZMCAEC-2011-009). In the first part for behavioral measurements, the rats were randomly divided into three groups (6 in each group): normal control group, sham-operated group and CCI group. In the first part for Western blot detection, the rats were randomly divided into 6 groups (6 in each group): sham group, 1, 3, 5, 7 and 14 days after CCI operation groups. In the second part of this study, the rats were randomly divided into six groups (6 in each group): normal control group, sham-operated group, CCI group, low dose of SB-657510 (10 μ g/20 μ L) group, middle dose of SB-657510 (30 μ g/20 μ L) group and high dose of SB-657510 (100 μ g/20 μ L) group. Finally, to observe the effects of the JNK inhibitor SP600125 and the NF- κ B inhibitor PDTC on neuropathic pain, the rats were randomly divided into five groups (6 in each group): normal control group, sham-operated group, CCI group, SP600125 (50 μ g/20 μ L) group and PDTC (20 μ g/20 μ L) group.

Neuropathic pain model

The chronic constriction injury of sciatic nerve was conducted according to the methods described by Bennett and Sacerdote (Bennett & Xie 1988, Sacerdote *et al.* 2008). Briefly, the sciatic nerve in the right thigh was exposed by blunt dissection under anesthesia with 10% chloral hydrate (300–350 mg/kg, intraperitoneal injection). Proximal to the sciatic's trifurcation, about 7 mm of nerve was free of adhering tissue and four ligatures (about 1 mm interval) with 4.0 chromic catgut were tied loosely around the sciatic nerve, until a brief twitch was observed. Sham surgery was produced by exposing the nerve without ligations. The surgical incision was completed by closing the muscles and skin in layers with 2.0 silk thread. All the operative procedures were performed by the same person.

Intrathecal catheters and drug treatment

For spinal drug administration, all male Sprague–Dawley (SD) rats were implanted with intrathecal (IT) catheters by inserting into the subarachnoid space between the L5 and L6 vertebrae (Milligan *et al.* 1999, Xu *et al.* 2009). After surgery, the rats were housed individually and had free access to water and food. The day after surgery, the rats were injected with 2% lidocaine in a volume of 30 μ L through catheters over 30 s, followed by a 10 μ L flush of physiological saline. Hind paw paralysis and/or paresis within 30 s and lasting 6–10 min indicated a successful catheterization (Shyu *et al.* 2012). Rats exhibiting postoperative neurologic deficits (approximately 25%) were excluded.

SB-657510 (Sigma) was dissolved in 1% dimethyl sulfoxide (DMSO). SB-657510 (10, 30 or 100 μ g) was injected consecutively once daily for 14 days through the intrathecal catheters in a volume of 20 μ L, followed by 10 μ L of 1% DMSO for flushing. The doses of SB-657510 were selected according to the previous study (Jarry *et al.* 2010) and our preliminary experiments. The animals in normal, sham and CCI groups received the same volume of 1% DMSO.

Behavioral analysis

Behavioral measurements were included of mechanical allodynia and thermal hyperalgesia. Both measurements were carried out 1 day before surgery and 1, 3, 5, 7 and 14 days after sciatic nerve injury. Rats were placed individually in a wire mesh cage and habituated for 30–60 min to allow acclimatization to the new environment (Tsuda *et al.* 2003, Tsuda *et al.* 2009). Calibrated Von Frey filaments (North Coast Medical, Inc, San Jose, CA, USA) were applied to the plantar surface of the rat hindpaw from below the mesh floor. The 50% mechanical withdrawal threshold (MWT) was determined using the up–down method (Chaplan *et al.* 1994).

Thermal hyperalgesia was assessed by measuring the latency of paw withdrawal in response to a radiant heat source. Thermal sensitivity was determined by the thermal withdrawal latency (TWL). Thermal punctuate stimuli was delivered to the plantar surface of the hind paw with a focused beam of radiant heat, and the withdrawal latency time was recorded. Result of each test was expressed as the mean of three withdrawal latencies in seconds. Five minutes was allowed between each test.

Rats were habituated to the test environment at least 30 min before the commencement of the experiments. Behavioral analysis was performed between 09:00 h and 12:00 h and was carried out in a quiet room.

Western blot analysis

In the first part for Western blot detection, at each time point of 1, 3, 5, 7 and 14 days after sciatic nerve injury, six rats were anesthetized, respectively, by 10% chloral hydrate (300–350 mg/kg, intraperitoneal injection), and the L4–6 spinal cord tissues were rapidly removed to measure the expressions of U-II and its receptor GPR14. In the second part, the rats were anesthetized and the L4–6 spinal cord tissues were collected at 14 days after sciatic nerve injury operation to measure the expressions of JNK and NF- κ B. Frozen spinal cord tissues were homogenized. The total and nuclear protein extracts were prepared using extraction kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. The protein content of crude was determined by the BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). The protein from each sample was separated on 10% sodium dodecyl sulfate–polyacrylamide gel and electrophoretically transferred onto PVDF membranes (Millipore) in Tris–glycine transfer buffer containing 20% methanol. The membranes were blocked with 3% BSA for 2 h and washed in Tris-buffered saline. Then the membranes were incubated at 4°C overnight with goat polyclonal anti-Urotensin II antibody (1:50 dilution; Santa Cruz, sc-21098), anti-GPR14 antibody (1:50 dilution; Santa Cruz, sc-10194), mouse anti-GAPDH antibody (1:1000 dilution; ZSGB-BIO, China), rabbit anti-phospho-JNK and anti-Total-JNK antibodies (1:1000 dilution; Cell Signaling Technology) or rabbit anti-NF- κ B p65 antibody (1:1000 dilution; Abcam). The immunoblots were subsequently washed and incubated in alkaline phosphatase-coupled rabbit anti-goat IgG antibody (1:1000 dilution, ZSGB-BIO), goat anti-rabbit IgG antibody (1:1000 dilution; ZSGB-BIO) or horse anti-mouse IgG antibody (1:1000 dilution; ZSGB-BIO) for 2 h. The bound antibodies were visualized using BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, China). Protein bands were then quantified using arbitrary units (AU) with the image analysis program, ImageJ (NIH, Bethesda, MD, USA). The expression of proteins was evaluated relative to GAPDH.

Immunofluorescent analysis

Rats were anesthetized by 10% chloral hydrate (300–350 mg/kg, intraperitoneal injection) and perfused transcardially with 100 mL phosphate-buffered saline (0.01 M PBS, pH 7.4), followed by 200 mL ice-cold 4% paraformaldehyde in 0.2 M phosphate buffer (300 mL, pH 7.4). The L4–6 spinal cord tissues from rats were removed immediately and post-fixed for 4–6 h at 4°C, and then cryoprotected by immersion for 24–48 h in sucrose gradients (5%, 10%, 15%, 20% and 30%) with 0.01 M PBS at 4°C. The spinal cord tissues were embedded with OCT, at –20°C and sectioned on a cryostat (Leica CM1900) at 30 µm in the transverse plane. The frozen sections were collected in PBS. After 3 washes in PBS, sections were incubated in PBS with 0.3% Triton X-100 (PBST) for 48 h at 4°C and then incubated with primary antibodies of rabbit anti-phospho-JNK (1:100 dilution; Cell Signaling Technology) or rabbit anti-phospho-NF-κB (1:100 dilution; Cell Signaling Technology). After rinsing in PBS, sections were incubated with the second antibodies solution in the darkness for 2 h at room temperature. Finally, sections were rinsed, mounted and cover-slipped with glycerol containing 2.5% anti-fading agent 1,4-di-aza-bi-cyclo-2,2,2-octane (DABCO, Sigma). Tissue sections were examined using laser scanning confocal microscopy (TCS SP2, Leica). ImageJ (NIH) was used for image quantification and analysis.

ELISA for IL-1β, IL-6 and TNF-α

Rats were anesthetized by 10% chloral hydrate (300–350 mg/kg, intraperitoneal injection) and the L4–6 spinal cord tissues were rapidly removed. Then, the spinal cord tissues were homogenized and centrifuged in the ice-cold normal saline, and the supernatants were applied to analysis. The levels of IL-1β, IL-6 and TNF-α in the spinal cord tissues were determined by enzyme-linked immunosorbent assay (ELISA) based on the manufacturer's instructions (XITANG, China).

Statistical analysis

All values were expressed as the mean ± s.d. The significant difference between the groups was analyzed with SPSS 13.0 by repeated-measures two-way (two factors of group and time) analysis of variance (behavioral data) or one-way analysis of variance (Western blot, Immunofluorescence and ELISA), followed by Student–Newman–Keuls *post hoc* tests. Differences were considered statistically significant when $P < 0.05$.

Results

Decreased MWT and TWL in CCI rats

As shown in Fig. 1, there was no significant difference in all the three groups before CCI operation. However, in CCI rats, the hind paw exhibited marked sensitivity to mechanical and thermal stimulation. The MWT significantly decreased by day 3 and the TWL markedly decreased by day 1 after CCI operation when compared with sham-operated rats or normal control rats ($P < 0.05$). This increased sensitivity to mechanical and thermal stimuli continued through at least 14 days after CCI operation. The thermal hyperalgesia and tactile allodynia in the hind paw were progressive over the post-lesion period, with maximum seen at 7 days after sciatic nerve injury ($P < 0.01$). Compared with the normal control group, the sham operation had no significant effect on the MWT or the TWL throughout the experiment.

Figure 1

Changes in pain behaviors after CCI operation in rats. (A) Mechanical withdrawal threshold (MWT). (B) Thermal withdrawal latency (TWL). Normal, normal control group; Sham, sham-operated group; CCI, chronic constriction injury of sciatic nerve group. All values are expressed as the mean ± s.d. $n = 6$. * $P < 0.05$, ** $P < 0.01$, vs sham-operated group. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0255>.

Increased expression of U-II and GPR14 in CCI rats by Western blot analysis

The expression of U-II and GPR14 in rat spinal dorsal horn was investigated by Western blot analysis using specific antibodies. GAPDH (36 kDa) was used as an internal standard. As indicated in Fig. 2, U-II and GPR14 were detected in the spinal dorsal horns of rats as bands of approximately 18 and 60 kDa, respectively. Compared with sham group rats, the expression levels of U-II and GPR14 were significantly enhanced at day 5, 7 and

14 after CCI operation ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively).

Intrathecal injection of SB-657510 attenuated the neuropathic pain in CCI rats

As shown in Fig. 3, there was no significant difference in all the six groups before CCI operation. However, the MWT significantly decreased by day 3 and the TWL markedly decreased by day 1 after CCI operation compared with sham-operated rats ($P < 0.01$, $P < 0.05$, respectively). This increased sensitivity to mechanical and thermal stimuli were markedly attenuated by intrathecal injection of U-II receptor antagonist SB-657510. Compared with the CCI group, intrathecal injection of the middle of SB-657510 (30 μ g) significantly increased the MWT and the TWL at day 5, 7, 14 and day 1, 3, 5, 7 and 14 after

Figure 2

Western blot analysis of the protein expression of Urotensin II and its receptor GPR14 in the spinal dorsal horns of rats. GAPDH was used as an internal standard. (A) Representative bands of the Western blot. (B) Ratio of Urotensin II to GAPDH denotes the expression of Urotensin II protein. C, Ratio of GPR14 to GAPDH denotes the expression of GPR14 protein. Sham, sham-operated group; 1, 3, 5, 7 and 14 represent 1, 3, 5, 7 and 14 days after CCI operation, respectively. All values are expressed as the mean $\pm$ s.d. $n = 6$. * $P < 0.05$, ** $P < 0.01$, vs sham-operated group.

Figure 3

Effect of Urotensin II receptor antagonist SB-657510 on pain behaviors of rats after CCI operation. SB-657510 (10 μ g, 30 μ g, and 100 μ g) was administered by intrathecal injection in CCI rats consecutively once daily for 14 days. (A) Mechanical withdrawal threshold (MWT). (B) Thermal withdrawal latency (TWL). Normal, normal control group; Sham, sham-operated group; CCI, chronic constriction injury of sciatic nerve group; L, low dose of SB-657510 (10 μ g) group; M, medium dose of SB-657510 (30 μ g) group; H, high dose of SB-657510 (100 μ g) group. All values are expressed as the mean $\pm$ s.d. $n = 6$. * $P < 0.05$, ** $P < 0.01$, vs sham-operated group; $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$, vs CCI group. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0255>.

sciatic nerve injury ($P<0.05$, $P<0.01$, $P<0.01$, $P<0.01$, $P<0.01$, $P<0.01$, $P<0.01$, respectively). Similar results were attained by intrathecal injection of the high doses of SB657510 (100 μ g) ($P<0.01$).

Intrathecal injection of SB-657510 downregulated the levels of IL-1 β , IL-6 and TNF- α in the spinal dorsal horns of CCI rats by ELISA

Proinflammatory cytokines IL-1 β , IL-6 and TNF- α in the spinal dorsal horns of rats were detected by ELISA. As shown in Fig. 4, the levels of IL-1 β , IL-6 and TNF- α were markedly elevated in CCI rats compared with those in sham rats ($P<0.01$). However, compared with CCI rats, intrathecal injection of the middle and high doses of SB-657510 (30 μ g and 100 μ g) markedly downregulated the enhanced levels of IL-1 β , IL-6 and TNF- α induced by

Figure 4

Effect of Urotensin II receptor antagonist SB-657510 on the levels of proinflammatory cytokines in the spinal dorsal horns of CCI rats. SB-657510 (10 μ g, 30 μ g, and 100 μ g) was administered by intrathecal injection in CCI rats consecutively once daily for 14 days. The expression levels of IL-1 β (A), IL-6 (B) and TNF- α (C) in the spinal dorsal horns were determined by enzyme-linked immunosorbent assay (ELISA). Normal, normal control group; Sham, sham-operated group; CCI, chronic constriction injury of sciatic nerve group; L, low dose of SB-657510 (10 μ g) group; M, medium dose of SB-657510 (30 μ g) group; H, high dose of SB-657510 (100 μ g) group. All values are expressed as the mean $\pm$ s.d. $n=6$. ** $P<0.01$, vs sham-operated group; * $P<0.05$, ▲ $P<0.01$, vs CCI group.

CCI of sciatic nerve ($P<0.05$, $P<0.05$, $P<0.01$, $P<0.05$, $P<0.01$, $P<0.01$, respectively).

Intrathecal injection of SB-657510 suppressed the expression of p-JNK and NF- κ B p65 in the spinal dorsal horns of CCI rats by Western blot analysis

The expression levels of p-JNK and nuclear NF- κ B p65 in rat spinal dorsal horn tissues were further investigated by Western blot analysis. As indicated in Fig. 5, the p-JNK was detected as bands of 54kDa and 46kDa and the NF- κ B p65 was detected as band of 65kDa using protein-specific antibodies, respectively. The expression of both p-JNK and nuclear NF- κ B p65 in the CCI group was significantly increased compared with those in the sham group ($P<0.01$). However, the enhanced expression of both p-JNK and NF- κ B p65 induced by CCI of sciatic nerve was markedly suppressed by intrathecal injection of the middle and high doses of SB-657510 (30 μ g and 100 μ g) ($P<0.01$). In addition, the results showed that the expression of Total-JNK in CCI group was not markedly changed compared to that in sham group. It was also showed that U-II antagonist SB657510 did not significantly affect the expression of Total-JNK. The values of this semi-quantitative measure were expressed as ratios of JNK and NF- κ B p65 to internal standard GAPDH (36kDa).

Intrathecal injection of SB-657510 suppressed the expression of p-JNK and NF- κ B p65 in the spinal dorsal horns of CCI rats by immunofluorescent analysis

The immunofluorescent staining was performed to further investigate the expressions of p-JNK and NF- κ B p65 in spinal dorsal horn of rats. It was showed in Fig. 6 that CCI significantly induced increased expression of p-JNK and NF- κ B p65 in spinal dorsal horn of rats compared with those in the sham group ($P<0.01$). The enhanced expression of p-JNK and NF- κ B p65 triggered by CCI was markedly attenuated by the middle doses of SB-657510 (30 μ g) ($P<0.01$, $P<0.05$, respectively).

Intrathecal injection of SP600125 and PDTC alleviated the neuropathic pain in CCI rats

As shown in Fig. 7, there was no significant difference in all the five groups before sciatic nerve injury operation. However, the rats developed significant thermal hyperalgesia and mechanical allodynia after CCI

Figure 5

Western blot analysis demonstrates the effect of Urotensin II receptor antagonist SB-657510 on the protein expression of p-JNK (A), total-JNK (B) and NF-κB p65 (C) in the spinal dorsal horns of CCI rats. GAPDH was used as an internal standard. SB-657510 (10 μg, 30 μg, and 100 μg) was administered by intrathecal injection in CCI rats consecutively once daily for 14 days. The total or the nuclear proteins were extracted, and equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with protein-specific antibodies. Normal, normal control group; Sham, sham-operated group; CCI, chronic constriction injury of sciatic nerve group; L, low dose of SB-657510 (10 μg) group; M, medium dose of SB-657510 (30 μg) group; H, high dose of SB-657510 (100 μg) group. All values are expressed as the mean ± s.d. $n=6$. ** $P<0.01$, vs sham-operated group; ▲▲ $P<0.01$, vs CCI group.

operation compared to sham-operated rats. Intrathecal injection of the JNK inhibitor SP600125 and the NF-κB inhibitor PDTC markedly attenuated the increased sensitivity to mechanical and thermal stimuli in CCI rats. Compared with the CCI group, intrathecal injection of 50 μg SP600125 significantly increased the MWT and the TWL at day 5, 7 and 14 and day 1, 3, 5, 7 and 14 after sciatic nerve injury ($P<0.05$, $P<0.01$, $P<0.01$; $P<0.05$, $P<0.01$, $P<0.01$, $P<0.01$, respectively). Similar results were attained by intrathecal injection of the NF-κB inhibitor PDTC (20 μg) ($P<0.05$ and $P<0.01$).

Discussion

U-II, a 12 amino acid peptide, was first discovered in the teleost fish neurosecretory cells, which are located in the caudal portion of the spinal cord and project to a neurohemal gland called the urophysis (Egginger *et al.* 2006). It has been demonstrated that U-II exerts potent proinflammatory effects in the metabolic syndrome (Barrette & Schwertani 2012), cardiovascular disease (Hassan *et al.* 2005), hepatic disease (Liang *et al.* 2014) and so on (Johns *et al.* 2004, Tabur *et al.* 2015), followed by the generation of all kinds of proinflammatory cytokines including IL-1β, IL-6 and TNF-α interacting with U-II receptor GPR14. Although neuropathic pain has been known as a chronic inflammatory disease closely related to neuroglia cells activation and generation of a lot of proinflammatory factors, little is known about

the relationship between proinflammatory peptide U-II and neuropathic pain. The expression profiles of U-II and its specific receptor GPR14 in spinal dorsal horn of rat with neuropathic pain have never been reported. In this study, we demonstrated that the expression levels of U-II and GPR14 were markedly increased in rats with neuropathic pain induced by CCI of the sciatic nerve. Furthermore, our results showed that intrathecal U-II receptor antagonist SB-657510 clearly attenuated thermal hyperalgesia and mechanical allodynia, reduced the secretion of proinflammatory cytokines including IL-1β, IL-6 and TNF-α and inhibited the phosphorylation of JNK and the activation of NF-κB p65 in the spinal dorsal horns of CCI rats.

In the present study, we found that both U-II and its specific receptor GPR14 were expressed in the spinal dorsal horns of sham rats, whereas it was significantly increased in the CCI group. In addition, the expression levels of U-II and GPR14 correlated positively with the MWT and TWL in rats. All the results mentioned previously suggested that U-II/GPR14 signaling may play an important role in neuropathic pain.

Then, to further clarify the role of U-II/GPR14 signaling in the neuropathic pain, the effects of U-II receptor antagonist SB-657510 on the MWT and TWL were investigated in CCI rats. The results indicated that both MWT and TWL were significantly decreased in CCI rats, whereas intrathecal injection of SB-657510 at the doses of 30 μg and 100 μg clearly attenuated mechanical allodynia and thermal hyperalgesia. This suggested that

Figure 6

Immunofluorescent analysis demonstrates the effect of Urotensin II receptor antagonist SB-657510 on the expression of p-JNK and p-NF-κB in the spinal cords of rats. (A) Representative photomicrographs showing immunofluorescent staining for p-JNK and p-NF-κB. (B) Quantification of immunofluorescent intensity for p-JNK in the spinal cord. (C) Quantification of immunofluorescent intensity for p-NF-κB in the spinal cord. Sham, sham-operated group; CCI, chronic constriction injury of sciatic nerve group; M, medium dose of SB-657510 (30 μg) group. All values are expressed as the mean ± s.d. $n=6$. Scale bars = 50 μm. ** $P<0.01$, vs sham-operated group; ▲ $P<0.05$, ▲▲ $P<0.01$, vs CCI group. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0255>.

U-II may be involved in the neuropathic pain by acting on its specific receptor GPR14.

Previous studies have demonstrated that U-II in combination with its receptor exerted potent proinflammatory effects by generation of all kinds of proinflammatory cytokines including IL-1β, IL-6 and TNF-α, and these proinflammatory cytokines played an important role in the development and maintenance of neuropathic pain (Arruda *et al.* 1998, Sweitzer *et al.* 2001, Sung *et al.* 2004, Zelenka *et al.* 2005, Ledeboer *et al.* 2007). How about the effect of U-II receptor antagonist on the production of IL-1β, IL-6 and TNF-α in CCI rats? In the present study, it was found that the levels of IL-1β, IL-6 and TNF-α in the spinal cord of CCI rats were markedly increased by ELISA method and intrathecal administration with 30 μg and 100 μg SB-657510 significantly reduced the

Figure 7

Effects of JNK inhibitor SP600125 and NF-κB inhibitor PDTC on pain behaviors in CCI rats. SP600125 (50 μg) and PDTC (20 μg) were administered by intrathecal injection in CCI rats consecutively once daily for 14 days. (A) Mechanical withdrawal threshold (MWT). (B) Thermal withdrawal latency (TWL). Normal, normal control group; Sham, sham-operated group; CCI, chronic constriction injury of sciatic nerve group; SP600125, SP600125 (50 μg) group; PDTC, PDTC (20 μg) group. All values are expressed as the mean ± s.d. $n=6$. * $P<0.05$, ** $P<0.01$, vs sham-operated group; ▲ $P<0.05$, ▲▲ $P<0.01$, vs CCI group. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0255>.

production of these proinflammatory cytokines. These data supported that U-II/GPR14 signaling system may promote the formation of neuropathic pain by facilitating the release of proinflammatory cytokines IL-1β, IL-6 and TNF-α in spinal cord of CCI rats.

JNK, as a member of mitogen-activated protein kinases (MAPKs), plays an important role in regulating persistent pain sensitization via astrocyte mechanisms (Zhuang *et al.* 2006, Gao *et al.* 2009). It can be activated by different inflammatory mediators, such as TNF and IL-1, and the activation of JNK MAPK pathways also increases the synthesis of multiple inflammatory mediators (Gao & Ji 2008). NF-κB, a transcription factor, is present in central nervous system including neuron and glial (Niederberger & Geisslinger 2008) and is involved in the maintenance of neuropathic pain (Manning 2004, Sommer & Kress 2004). It is reported that the activation of NF-κB is necessary for transcription of the genes encoding the proinflammatory cytokines (Clavel & Haller 2007). To further investigate

the underlying mechanisms by which U-II receptor antagonist reduced the release of inflammatory cytokines, the effects of SB-657510 on the phosphorylation of JNK and the activation of NF- κ B p65 were explored in CCI rats. Although there was no significant difference for the level of Total-JNK expression in all the observed groups, the significant increase of JNK phosphorylation and NF- κ B p65 activation was observed after CCI of sciatic nerve, which paralleled the marked increase in the expression levels of IL-1 β , IL-6 and TNF- α . However, the phosphorylation of JNK and the activation of NF- κ B p65 were markedly attenuated by intrathecal administration of 30 μ g and 100 μ g SB-657510 in CCI rats, and this attenuation was followed by a decrease in IL-1 β , IL-6 and TNF- α expression levels. These results indicated that the suppression of JNK phosphorylation and NF- κ B p65 activation after CCI by the intrathecal administration of SB-657510 and the subsequent reduction in the secretion of proinflammatory cytokines, such as IL-1 β , IL-6 and TNF- α , may be a potential mechanism for the regulatory effects of U-II receptor antagonist on neuropathic pain. To further confirm the opinion above, the effects of JNK inhibitor SP600125 and NF- κ B inhibitor PDTC on neuropathic pain were investigated. The results showed that intrathecal injection of SP600125 and PDTC significantly attenuated thermal hyperalgesia and mechanical allodynia induced by CCI of sciatic nerve. These similar results of SP600125 and PDTC to U-II antagonist SB657510 further demonstrated that the analgesic effects of U-II antagonist were possibly mediated by the inhibition of JNK phosphorylation and NF- κ B activation.

However, there are still some limitations of our study. Although U-II receptor antagonist SB-657510 can attenuate the enhanced spinal inflammation induced by CCI of sciatic nerve, whether the effect of SB-657510 was mediated by acting on neurons, astrocytes and (or) microglia remains unclear. Therefore, further studies are needed to clarify the target cells of U-II regulation.

Taken together, our present study indicates that the expression levels of both U-II and its specific receptor GPR14 are significantly upregulated in the spinal dorsal horns of rats with neuropathic pain induced by chronic constriction injury of sciatic nerve. Moreover, U-II receptor antagonist SB-657510 could significantly attenuate thermal hyperalgesia and mechanical allodynia by the suppression of JNK phosphorylation and NF- κ B p65 activation as well as subsequent reduction of proinflammatory cytokines in the spinal dorsal horns of CCI rats. These findings suggest that U-II receptor antagonist has a potential to alleviate neuropathic pain.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Jing Li helped conduct the study, analyze the data and prepare the manuscript; Pan-Pan Zhao helped conduct the study, analyze the data and prepare the manuscript; Ting Hao helped prepare the manuscript; Dan Wang helped prepare the manuscript; Yu Wang helped prepare the manuscript; Yang-Zi Zhu helped prepare the manuscript. Yu-Qing Wu helped design the study, analyze the data and prepare the manuscript. Cheng-Hua Zhou helped design the study, analyze the data and prepare the manuscript.

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