Int J Clin Exp Med 2015;8(7):11202-11207
www.ijcem.com /ISSN:1940-5901/IJCEM0009459

Original Article

Analgesic effect of TAK-242 on neuropathic pain in rats

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Received April 22, 2015; Accepted June 21, 2015; Epub July 15, 2015; Published July 30, 2015

Abstract: Background: The current study investigated the analgesic effect of the Toll-like receptor 4 (TLR4) specific antagonist TAK-242 on neuropathic pain in rats and its underlying mechanism. Methods: A total of 132 adult Sprague-Dawley (SD) rats were randomly divided into four groups: the sham operation group, the neuropathic pain model group, the TAK-242 low-dose treatment group, and the TAK-242 high-dose treatment group. The heat pain and mechanic pain thresholds of rats were detected on preoperative day 1 and postoperative days 1, 3, 7, and 10. The expression levels of IκBα, p65, IL-1β, and TNF-α in the spinal cord dorsal horn were detected on postoperative day 7 in one group of rats. Results: Compared with rats in the sham operation group, the heat pain and mechanic pain thresholds of the rats in the neuropathic pain model group significantly decreased; their expression levels of p65, IL-1β, and TNF-α significantly increased; and their expression level of IκBα significantly decreased. Compared with the neuropathic pain group, high doses of TAK-242 significantly inhibited the expression of p65, IL-1β, and TNF-α; significantly increased the expression level of IκBα; and upregulated the heat pain and mechanic pain thresholds. Conclusion: TAK-242 might improve neuropathic pain through downregulation of the NF-κB pathway.

Keywords: Neuropathic pain, TAK-242

Introduction

Neuropathic pain originates from the central nervous system. Because the pathogenic mechanism of neuropathic pain is relatively complex, some clinical patients currently have inferior therapeutic effects that severely influence their quality of life [1]. Therefore, the development of new drugs for neuropathic pain, which are effective and have few side effects, has important clinical significance. Studies have shown that the Toll-like receptor 4 (TLR4)/NF-κB signaling pathway is an important participant in the mechanism of neuropathic pain. Many drugs and measures that downregulate the TLR4/NF-κB signaling pathway can effectively exert an analgesic effect [2-4]. TAK-242 is a specific TLR4 antagonist and can effectively pass through the blood-brain barrier. The current study investigated the analgesic effect of TAK-242 and its underlying mechanism to provide new thinking and methods for treating neuropathic pain.

Materials and methods

Materials

The Experimental Animal Medical Center of Nanjing Medical University provided male Sprague-Dawley (SD) rats with body weights ranging from 200-330 g. Nuclear protein and total protein extraction solutions were purchased from Beyotime Biotechnology (Nantong, China). IκB, p65, and TNF-α antibodies were purchased from Santa Cruz Biotechnology (USA). IL-1β antibody was purchased from Abcam (USA). TAK-242 was purchased from Tocris (USA). TNF-α and IL-1β enzyme-linked immunosorbent assay (ELISA) detection reagent kits were purchased from Multisciences (China).

Experimental grouping and the establishment of the neuropathic pain model in rats

Male SD rats were randomly divided into four groups: the sham operation group, the neuropathic pain group, the neuropathic pain + TAK-

Table 1. Heat pain thresholds of each group at different time points (n = 12 in eachgroup)

Item	Number	Preoperative day 1	Postoperative day 1	Postoperative day 3	Postoperative day 7	Postoperative day 10
Blank group	20	22.1 ± 1.63	22.6 ± 2.13	22.7 ± 1.78	22.1 ± 2.01	22.8 ± 1.97
Model group	20	22.7 ± 1.73	15.6 ± 1.76*	14.1 ± 1.65*	8.7 ± 1.98*	8.5 ± 2.01*
Low-dose group	20	22.6 ± 1.58	17.9 ± 1.68	18.6 ± 1.39	19.7 ± 2.32#	19.2 ± 2.43
High-dose group	20	22.5 ± 1.65	18.5 ± 2.01#	19.8 ± 2.31#	22.9 ± 2.41#	22.1 ± 2.51#

*$P < 0.05$ compared with the blank control group; #$P < 0.05$ compared with the model group.

Table 2. Mechanic pain threshold of each group at different time points (n = 12 ineach group)

Item	Number	Preoperative day 1	Postoperative day 1	Postoperative day 3	Postoperative day 7	Postoperative day 10
Blank group	20	59.6 ± 2.11	59.6 ± 3.22	59.6 ± 3.22	59.6 ± 3.21	58.6 ± 3.22
Model group	20	59.9 ± 2.21	40.8 ± 4.51*	25.3 ± 3.71*	22.6 ± 3.12*	25.6 ± 3.13*
Low-dose group	20	59.4 ± 2.13	44.1 ± 3.92	30.3 ± 4.54#	31.1 ± 3.91#	35.1 ± 3.94#
High-dose group	20	59.1 ± 2.31	44.7 ± 4.17#	45.1 ± 4.33#	49.7 ± 3.87#	50.1 ± 3.82#

*$P < 0.05$ compared with the blank control group; #$P < 0.05$ compared with themodel group.

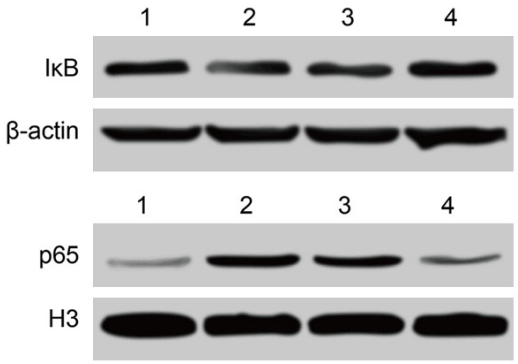

Figure 1. Western blot results of IκBα and p65 in each group (1: Sham operation group, 2: Model group, 3: Low dose group, 4: High dose group).

242 (low-dose) group, and the neuropathic pain + TAK-242 (high-dose) group. The establishment of the neuropathic pain model was based on a previous report [5]. A chronic neuropathic pain model was established in rats using the chronic sciatic nerve compression injury method. Rats were anaesthetized via an intraperitoneal injection of 10% chloral hydrate at 400 mg/kg. After anaesthetization, rats were disinfected and draped, and the sciatic nerve trunk was separated from the surrounding soft tissues. The sciatic nerve was ligated using a 4.0 catgut suture via the slight compression of the epineurium to cause mild tremor in the calf muscles for a total of four ligations at an interval of 1 mm. The muscle and skin were sutured layer by layer. In the sham operation group, the sciatic nerve was only exposed without ligation treatment; the other treatments matched that of the model group. The same technician established the model in each rat. Intraperitoneal injections of TAK-242 (5 mg/kg and 10 mg/kg) were conducted in each group from preoperative day 1 to postoperative day 7, once per day. The dosage was based on previous literature [6, 7]. Changes in the pain behavior of the rats in each group were observed before the operation and on postoperative days 1, 3, 7, and 10. The protein expression in the spinal cord on postoperative day 7 was observed in another group of rats. The protein expression levels of IL-1β and TNF-α in the spinal cord tissues 7 days after the successful establishment of the model were detected using ELISA. In addition, after the pain threshold was detected on day 7, five animals from each group were perfused for sample collection. The expression levels of IκBα, p65, TNF-α, and IL-1β from the spinal cord tissues of rats were measured using immunohistochemistry.

Detection of the heat pain and mechanic pain thresholds

Basic pain threshold values using the paw withdrawal mechanical threshold values and the thermal withdrawal latency behavior were evaluated on rats after grouping. The thermal withdrawal latency of the hind foot at the nerve liga-

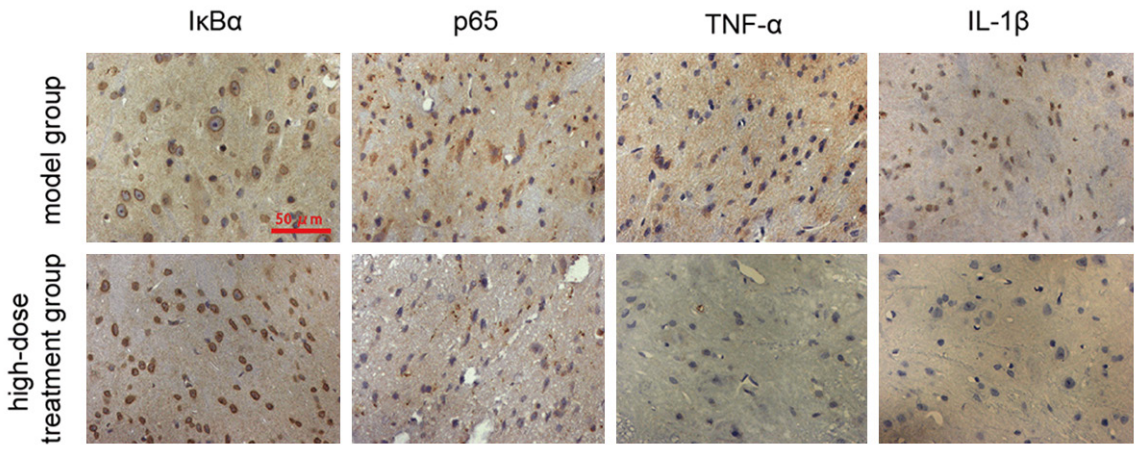

Figure 2. IκBα, p65, IL-1β, and TNF-α immunochemistry result of the model group and the high-dose treatment group.

Table 3. Analysis of the expression levels of IκBα, p65, IL-1β, and TNF-α in eachgroup.

Item	n	IκBα	p65	IL-1β	TNF-α
		(IκBα/β-actin)	(p65/H3)	(pg/mg)	(pg/mg)
Blank group	10	0.86 ± 0.07	0.02 ± 0.01	10.2 ± 2.5	2.9 ± 0.9
Model group	16	0.42 ± 0.03*	0.43 ± 0.12*	42.9 ± 6.3*	13.8 ± 3.2*
Low-dose group	10	0.59 ± 0.05	0.39 ± 0.09	31.5 ± 5.4	11.3 ± 3.1
High-dose group	16	0.92 ± 0.13#	0.11 ± 0.03#	22.6 ± 4.3#	5.1 ± 2.7#

*$P < 0.05$ compared with the blank control group; #$P < 0.05$ compared with themodel group.

tion side was measured using a thermal pain stimulator. The paw withdrawal mechanical threshold value of the hind foot at the nerve ligation side (i.e., the right side) was measured using the Von Frey filament method. The specific evaluation methods referenced a previous report [3].

Western blot

Seven days after the model was successfully established, rats were anaesthetized, and the thoracic cavity was opened. Normal saline (80 mL) was perfused through the left heart apex. An incision at the back was made to expose L4-6. The vertebral plate was removed, and spinal cord tissues were collected and stored in liquid nitrogen for future use. Spinal cord tissues at 80 mg were collected from each rat, and a tissue lysis buffer was added at the ratio of 1:1,000. After being fully ground, the tissues were centrifuged at 12,000 r/min for 10 min. The supernatant was collected, added into 5 × loading buffer at the ratio of 4:1, and heated in boiling water for 10 min. Electrophoresis was performed with 35 μg protein in each well. Proteins were transferred onto a membrane and blocked in 5% milk at room temperature for 1 h. The membrane was incubated in the diluted primary antibody solution at 4°C overnight. After being washed with TBST, the membrane was incubated with the diluted secondary antibody solution at room temperature for 1 h. After being washed with TBS, a developing solution was added for development. The results were quantified using Image J software.

Immunohistochemistry

Seven days after the successful establishment of the model, rats were anaesthetized using chloral hydrate, perfused using 100 mL normal saline through the left ventricle, and fixed via perfusion with 4% paraformaldehyde. The spinal cord tissue specimen at the L4-6 segments was embedded in paraffin for serial sections at a thickness of 6 μm. The expression levels of IκBα, p65, IL-1β, and TNF-α were detected using immunohistochemistry. The immunohistochemistry reagent kit was purchased from ZSGB-BIO. The manipulation procedure was performed strictly according to the instruction manual.

Detection of the expression levels of TNF-α and IL-1β using ELISA

Seven days after the neuropathic pain model was successfully established, rats were anaesthetized, and normal saline was perfused through the left heart apex. The L4-6 segments of the spinal cord tissue were collected, fully lysed, and centrifuged. The supernatant was collected and detected using ELISA. The ELISA detection reagent kit was purchased from Roche (USA).

Statistical analyses

The experimental data were processed using SPSS 17.0. The data are presented as means ± standard deviations ($\bar{x} \pm s$). The neurological function scores were examined using the Kruskal-Wallis test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA). $P < 0.05$ indicated statistical significance.

Results

The effect of TAK-242 on the heat pain and mechanic pain thresholds of the rats in the neuropathic pain groups

The basic paw thermal withdrawal latency values and the basic mechanic pain threshold values are shown in **Tables 1** and **2**. The results on preoperative day 1 showed that the basic paw thermal withdrawal latency values and the basic mechanic pain threshold values did not significantly differ among groups ($P > 0.05$). Compared with the rats in the sham operation group, the heat pain and mechanic pain thresholds of the rats in the neuropathic pain group on postoperative days 1, 3, 7, and 10 significantly decreased ($P < 0.05$). Compared with the rats in the neuropathic pain group, TAK-242 significantly improved the heat pain and mechanic pain thresholds in rats in a dose-dependent manner. High doses of TAK-242 significantly increased the heat pain and mechanic pain thresholds of rats with neuropathic pain ($P < 0.05$).

The effect of TAK-242 on the protein expression of IκBα and NF-κB p65

As shown in **Figures 1**, **2** and **Table 3**, the expression levels of IκBα significantly decreased and those of NF-κB p65 protein significantly increased in the spinal cord tissues of rats with neuropathic pain on postoperative day 7 compared with those in the sham operation group ($P < 0.05$). High doses of TAK-242 significantly increased the protein expression levels of IκBα but inhibited the expression levels of p65 in the cell nucleus ($P < 0.05$). Immunohistochemistry results also confirmed the Western blot results (**Figure 2**).

The effect of TAK-242 on the expression of inflammatory factors, TNF-α and IL-1β

The expression levels of TNF-α and IL-1β in the spinal cord tissues of the neuropathic pain rats on postoperative day 7 were significantly increased compared with those of the rats in the sham operation group ($P < 0.05$; **Table 3** and **Figure 2**). The expression of TNF-α and IL-1β was significantly inhibited in the high-dose group ($P < 0.05$; **Table 3** and **Figure 2**).

Discussion

TLR4 is a type I transmembrane protein [8] and consists of three parts: the extracellular domain, the transmembrane domain, and the intracellular domain. The transduction of the TLR4 signaling pathway primarily includes the MyD88-independent and dependent pathways. The former does not depend on the activation of the MyD88 protein; rather, it primarily depends on the IRF-3 and delayed NF-κB responses. The latter primarily depends on the activation of MyD88 to induce rapid NF-κB responses; the activation of NF-κB can regulate the production of pro-inflammatory factors such as IL-1β, IL-6, Copx-2, and iNOS. These molecules can further promote the activation of microglial cells and the production of inflammatory mediators to function in the nociceptive pathways, thereby eventually resulting in the development and maintenance of pain [9-11]. Several previous studies have shown that the mRNA and protein expression levels of TLR4 in spinal cord microglial cells increased significantly in neuropathic pain animal models. TLR4 gene knockout significantly reduces microglial activation and the expression of pain related factors and significantly relieves the severity of neuropathic pain. Treatment with TLR4 anti-sense oligonucleotides and the specific antagonist FP-1 [12] might also relieve the severity of neuropathic pain. The above study results all indicate that TLR4 is an important participant in the occur-

rence and development of neuropathic pain. Drugs that target TLR4 might have significance with regard to the prevention and treatment of neuropathic pain.

TAK-242 is a specific antagonist of TLR4 and can effectively pass through the brain-blood barrier. Recent studies have shown that TAK-242 can inhibit the NF-κB signaling pathway to exert significant nerve protection functions with regard to acute damage of the central nervous system (e.g., cerebral ischemia-reperfusion injury and traumatic brain damage) [6]. The present study showed that a low-dose TAK-242 treatment did not have an obvious treatment effect on neuropathic pain; however, the high-dose treatment significantly improved neuropathic pain. This result indicates that the treatment effect of TAK-242 on neuropathic pain behaved in a dose-dependent manner. Therefore, in this mechanism study, we primarily used a high dose of TAK-242 to investigate relevant mechanisms. The results showed that treatment via a high dose of TAK-242 does not effectively downregulate TLR4 protein expression because TAK-242 is an antagonist (but not an inhibitor) of TLR4. This finding is consistent with previous studies. Additional studies have shown that TAK-242 effectively decreases p65 expression in the nucleus of spinal cord dorsal horn nerves in rats with neuropathic pain and downregulates the expression of the downstream inflammatory related factors IL-1β and TNF-α. Due to the function of inflammatory factors in neuropathic pain, we speculate that TAK-242 might also downregulate the expression of downstream-related inflammatory factors to exert analgesic effects through the inhibition of the TLR4/NF-κB signaling pathway. TAK-242 has been applied in a phase III clinical trial to treat sepsis [13] and has demonstrated excellent safety in the human body, suggesting that TAK-242 could be used as a new drug to treat neuropathic pain.

In summary, TAK-242 can effectively relieve the severity of neuropathic pain. Its function might be associated with the inhibition of the expression of NF-κB and its downstream inflammatory factors IL-1β and TNF-α.

Acknowledgements

We would like to acknowledge the reviewers for their helpful comments on this paper.

Disclosure of conflict of interest

None.

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