Original Article

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A single dose of ketamine relieves fentanyl-induced-hyperalgesia by reducing inflammation initiated by the TLR4/NF-κB pathway in rat spinal cord neurons

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SUMMARY A large amount of clinical evidence has revealed that ketamine can relieve fentanyl-induced hyperalgesia. However, the underlying mechanism is still unclear. In the current study, a single dose of ketamine (5 mg/kg or 10 mg/kg), TAK-242 (3 mg/kg), or saline was intraperitoneally injected into rats 15 min before four subcutaneous injections of fentanyl. Results revealed that pre-administration of ketamine alleviated fentanyl-induced hyperalgesia according to hind paw-pressure and paw-withdrawal tests. High-dose ketamine can reverse the expression of toll-like receptor-dimer (d-TLR4), phospho- nuclear factor kappa-B (*p*-NF-κB, *p*-p65), cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) 1 d after fentanyl injection in the spinal cord. Moreover, fentanyl-induced-hyperalgesia and changes in the expression of the aforementioned proteins can be attenuated by TAK-242, an inhibitor of TLR4, as well as ketamine. Importantly, TLR4, *p*-p65, COX-2, and IL-1β were expressed in neurons but not in glial cells in the spinal cord 1 d after fentanyl injection. In conclusion, results suggested that a single dose of ketamine can relieve fentanyl-induced-hyperalgesia *via* the TLR4/NF-κB pathway in spinal cord neurons.

Keywords fentanyl, hyperalgesia, ketamine, neuron, NF-κB, TLR4, COX-2, TNF-α

1. Introduction

Fentanyl has been widely used perioperatively as an analgesic since it was first synthesized in 1960. However, the use of high-dose fentanyl can induce hyperalgesia simultaneously from several hours to a few days after infusion (*1-4*). Fentanyl-induced hyperalgesia (FIH) decreases patients' quality of life and requires larger doses of fentanyl for pain relief, which increases adverse reactions such as nausea, vomiting, and respiratory depression. Thus, reducing FIH in patients is important.

For several decades, *N*-methyl-D-aspartate (NMDA) antagonists have been found to prevent opioid-induced hyperalgesia (OIH) and morphine tolerance (*5*). Ketamine, a nonselective NMDA antagonist, has clinically been used as an analgesic in acute pain, chronic pain, and cancer pain management (*6*). Ketamine was also found to play an effective role in decreasing remifentanil and fentanyl-induced hyperalgesia in many clinical trials (*7, 8*). In animal models of long-term hyperalgesia induced with chronic morphine injections (*9*) or acute repeated fentanyl injections (*10*), ketamine demonstrated significant preventive effects. However, there is still a lack of molecular evidence for a specific mechanism for the ability of ketamine to prevent FIH. Is only the NMDA receptor associated with its action? Or do other molecular mechanisms contribute to it?

Studies have demonstrated that TLR4 activation

contributed to opioid-induced tolerance and hyperalgesia and that the TLR4 antagonist LPS-RS can prevent OIH and tolerance. Recently, COX-2 and prostaglandin E2 (PGE2) in the spinal cord were found to be closely associated with FIH (*11*). Given the aforementioned findings, the current study investigated the effect of a single dose of ketamine on FIH and found that it alleviates FIH through the TLR4 receptor and its downstream pathway in the spinal cord.

2. Materials and Methods

2.1. Animals

Male Sprague Dawley (SD) rats weighing 200-220 g were purchased from the Laboratory Animal Center of Sun Yat-sen University. The rats were housed under conditions of a controlled temperature (24±1°C) and humidity (50-60%) with free access to sterile water and standard laboratory chow. Animals were kept under a 12 h:12h light /dark cycle.

2.2. Ethics

Ethical approval for this study was provided by the Ethical Committee of Guangdong Provincial People's Hospital, Guangdong, China (Chairperson Prof Hong, Tan) on November 29, 2021 (approval No.: KY-Q-2021-234-01). All data in the study were collected and analyzed between December 2021 and February 2023.

2.3. Drug administration

In accordance with a previous study (*12*), fentanyl (Yichang Humanwell Pharmaceutical Co., Ltd., Hubei, China) and ketamine (Heng Rui Pharmaceuticals, Jiangsu, China) were dissolved in saline. Ketamine 5 mg/kg or 10 mg/kg was respectively injected intraperitoneally into the rats, which were divided into a low-dose ketamine group (LK) and high-dose ketamine (HK). Control animals (Con) received the same volume of saline intraperitoneally.

Fentanyl was dissolved with saline to a concentration of 0.05 mg/mL and was injected subcutaneously 4 times at the dose of 60 μg/kg at 15-min intervals. Low-dose or high-dose ketamine was intraperitoneally injected 15 min before the fentanyl injection, and groups were designated low-dose ketamine + fentanyl (LKF) or high-dose ketamine + fentanyl (HKF). The fentanyl group was injected with the same volume of saline intraperitoneally 15 mins before the fentanyl injection. The control group received the same volume of saline subcutaneously and intraperitoneally.

TAK-242 (MedChemExpress, NJ, USA), a TLR4 inhibitor, was dissolved in 0.1% dimethyl sulfoxide (DMSO) with normal saline. Rats received 3 mg/kg TAK-242 intraperitoneally 15 mins before the fentanyl injection and were designated the TAK-242 + Fen group. The Fen group received the same volume of 0.1% DMSO intraperitoneally 15 mins before the fentanyl injection.

2.4. Experimental design

The experiment was conducted as shown in Figure 1. In part I, rats were randomly assigned to one of three groups to observe the influence of a single injection of ketamine: the LK group received an intraperitoneal injection of low-dose ketamine (5mg/kg) while the HK group received high-dose ketamine (10mg/kg). As a control, the Con group that was injected with saline intraperitoneally. The thermal hyperalgesia test and the Randall Selitto test were conducted 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, and 1 d after the injection. The lumbar enlargement of the spinal cord (L4-L6) was harvested on day 1 for Western blotting.

To study the preventive effect of a single injection of ketamine on hyperalgesia induced by repeated subcutaneous injection of fentanyl, the rats were randomly assigned into four groups in part II: the Fen group was given four injections at 15-min intervals; the LKF group or HKF group received low-dose (5 mg/kg) or high-dose (10 mg/kg) ketamine 15 min before the first injection of fentanyl; and the Con group was given the same volume saline intraperitoneally and subcutaneously. The rats were subjected to the thermal hyperalgesia test and Randall Selitto test 15 min, 30 min, 1 h, 4 h, 1 d, and 7 d after the first administration of fentanyl. Western blotting and immunohistochemistry were performed on the rats on day 1 and day 7 after anesthesia.

Part III was to study whether blocking TLR4 signaling with TAK-242 could directly alleviate hyperalgesia and related proteins changes caused by fentanyl. TAK-242 was intraperitoneally 15 min before the fentanyl injection in the TAK-242 + Fen group while the Fen group received fentanyl. One day later, behavioral tests were conducted and molecules were detected.

2.5. Behavior test

In accordance with the method described previously (*13*), the thermal nociceptive test was conducted with a plantar test apparatus (IITC Life Science, CA, USA). In brief, rats were individually placed in a plexiglass chamber on a colorless transparent glass platform for 30-min for acclimation. A light beam was directed toward the middle plantar aspect of the left hind paw near the toes. The intensity of the light beam was 30 W and a 20-s cut-off time was used to avoid additional thermal injury. Three measurements were made per rat in each group with a 5-min interval. The paw withdrawal latency was recorded as the length of time (s) between projection of the light beam and paw withdrawal.

The mechanical pain threshold was measured with a mechanical pain threshold apparatus (UGO BASILE, Italy) and used as an indicator of mechanical nociception (*14, 15*). Stimulus in the form of mechanical pressure was applied to the midpoint of the right hind paw, and pressure was increased linearly. Three measurements were made per rat in each group with a 5-min interval. The pressure intensity (g) that caused an escape reaction was determined as the mechanical pain threshold.

2.6. Western blotting

Rats were euthanatized with 20% urethane (MACKLIN, Shanghai, China) 1.6 g/kg intraperitoneally. The lumbar enlargement of the spinal cord (L4-L6) was exposed by laminectomy. The dorsal half of the spinal cord was harvested in liquid nitrogen and then frozen at -80℃ until use. The cytoplasm and membrane protein were extracted following the protocol in the kit (Thermo Fisher Scientific, MA, USA). Cytoplasm protein was separated with TGX gel (Bio-rad, CA, USA), transferred onto a PVDF membrane (Merk Millipore, MA, USA), and incubated with antibodies against TLR4 (Novus Biologicals, USA), *p*-p65 (Cell Signaling Technology, MA, USA) COX-2 (Abcam, MA, USA), IL-1β (Abcam, MA, USA), TNF-α (Abcam, MA, USA), NaK (Abcam, MA, USA), β-action (CST, MA, USA), and GAPDH (CST, MA, USA). Blots were washed and incubated in HRP-linked anti-rabbit IgG antibody (KPL, MD, USA) and HRP-linked anti-mouse IgG antibody (KPL, MD, USA). Protein blots were visualized using Clarity ECL Substrates (Merk Millipore, MA, USA).

2.7. Immunohistochemistry

Rats were anesthetized with 20% urethane (MACKLIN, Shanghai, China) 1.6 g/kg intraperitoneally, and saline was perfused through the ascending aorta followed by 4% paraformaldehyde (PFA) (Sigma-Aldrich MO, USA). The lumbar enlargement of the spinal cord (L4-L6) was rapidly dissected and postfixed in 4% PFA. After dehydration in 30% sucrose, the samples were cut transversely and consecutively into slices of 20 μm and processed for immunohistochemistry with antibodies against TLR4, *p*-p65 (Affinity Bioscience, OH, USA), COX-2, IL-1β, NeuN (Merk Millipore, MA, USA), Iba-1 (Abcam, MA, USA), and GFAP (Abcam, MA, USA). Following incubation at 4℃ overnight, the slices were finally incubated with Cy-3-conjugated (Jackson Immunoresearch Laboratories, PA, USA), 488-conjugated (Invitrogen, CA, USA), and 647-conjugated (Invitrogen, CA, USA) secondary antibodies for 1 h at room temperature and then mounted on coverslips with Fluoromount-G with DAPI (Southern Biotech, Birmingham, AL). Images were captured with the LSM 780 confocal microscope (Zeiss, Germany).

2.8. Statistical analysis

All data were analyzed using GraphPad Prism 8 (GraphPad Software, USA) and are expressed as the mean ± SD. Nociceptive thresholds or latencies were quantified as direct measurement data and the area under the response time curve (AUC). Behavior test data and Western Blotting data were analyzed with one-way analysis of variance (ANOVA) or two-way ANOVA followed by Tukey's *post hoc* test. In all cases, $p < 0.05$ was considered to be a statistically significant difference.

3. Results

3.1. A single injection of ketamine relieves mechanical allodynia and thermal hyperalgesia induced by fentanyl

Ketamine is clinically used as an anesthetic, so to exclude the influence of a single low (5 mg/kg) or high dose (10 mg/kg) of ketamine on animals, hyperalgesia and allodynia were measured 15 min to 1 d after the ketamine injection (Figure 1A). As shown in Figure 2, both high and low doses of ketamine increased thermal hyperalgesia (Figure 2A) compared to the control group. The thermal pain threshold 30 min after injection (high dose: $p = 0.0384$ and low dose: $p = 0.0479$) and the mechanical pain threshold (Figure 2B) 15 min after injection (low dose: $p = 0.0433$ and high dose: $p < 0.001$) increased significantly, and the analgesic effect disappeared 1 h after injection. Western blotting revealed that ketamine had no effect on TLR4 (Figure 2C), *p*-p65 (Figure 2D), COX-2 (Figure 2E), IL-1β (Figure 2F), or TNF-α (Figure 2G) in the spinal cord on day 1. These results indicate that ketamine has a transient acute analgesic effect within 15-30 min but the animals' pain threshold returned to normal after 1 h, suggesting that ketamine has no significant long-term analgesic effect.

To determine if a single injection of ketamine is capable of reducing the hyperalgesia induced by fentanyl, mechanical and thermal pain thresholds were measured after the last injection of fentanyl (Figure 1B). The Hargreaves test (Figure 3A) revealed that the Fen ($p < 0.001$), LKF ($p < 0.001$), and HKF ($p < 0.001$) groups had a significantly increased paw withdrawal threshold 1-2 h after drug administration and returned to normal 4 h later. These may result from the rapid and strong analgesic effect of fentanyl. Importantly, the Fen group displayed thermal hyperalgesia from day 1 to day 2 compared to the control group (day 1: $p = 0.0478$, day 2: $p = 0.0254$), while the LKF (day 2: $p = 0.0065$) and HKF (day 2: $p = 0.0081$) groups displayed a higher paw withdrawal threshold compared to the Fen group and did not differ from the control group on day 2. Statistics indicated that the AUCs for the Fen ($p < 0.001$), LKF ($p < 0.001$), and HKF ($p < 0.001$) groups were larger than that for the control group 1 to 4 h after drug administration in the Hargreaves test (Figure 3B).

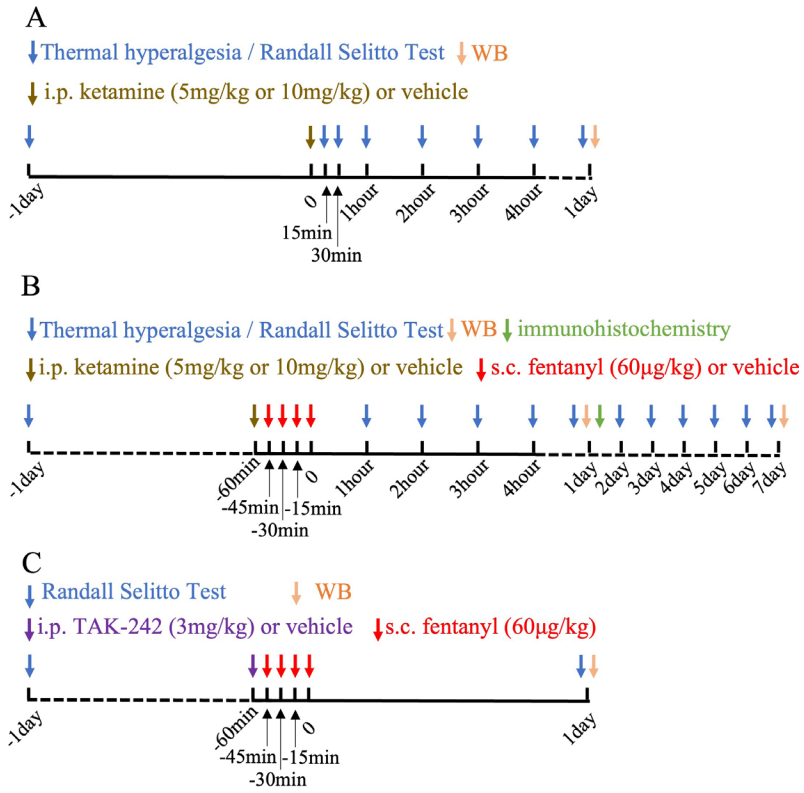

Figure 1. Timeline of the experiments. **(A)** One day after the behavior test for the baseline, low-dose and high-dose ketamine was injected into rats intraperitoneally. Then, behavior tests and WB were performed from 1 h to 1 d after the ketamine injection. **(B)** One d after the behavior test for the baseline, low-dose and high-dose ketamine was injected respectively 15 mins before 4 fentanyl injections at 15-min intervals. The behavior tests, WB, and immunohistochemistry were performed after the final ketamine injection. **(C)** One d after the behavior test for the baseline, TAK-242 was injected 15 mins before 4 fentanyl injections at 15-min intervals. Then, behavior tests and WB were performed 1 d after the final fentanyl injection.

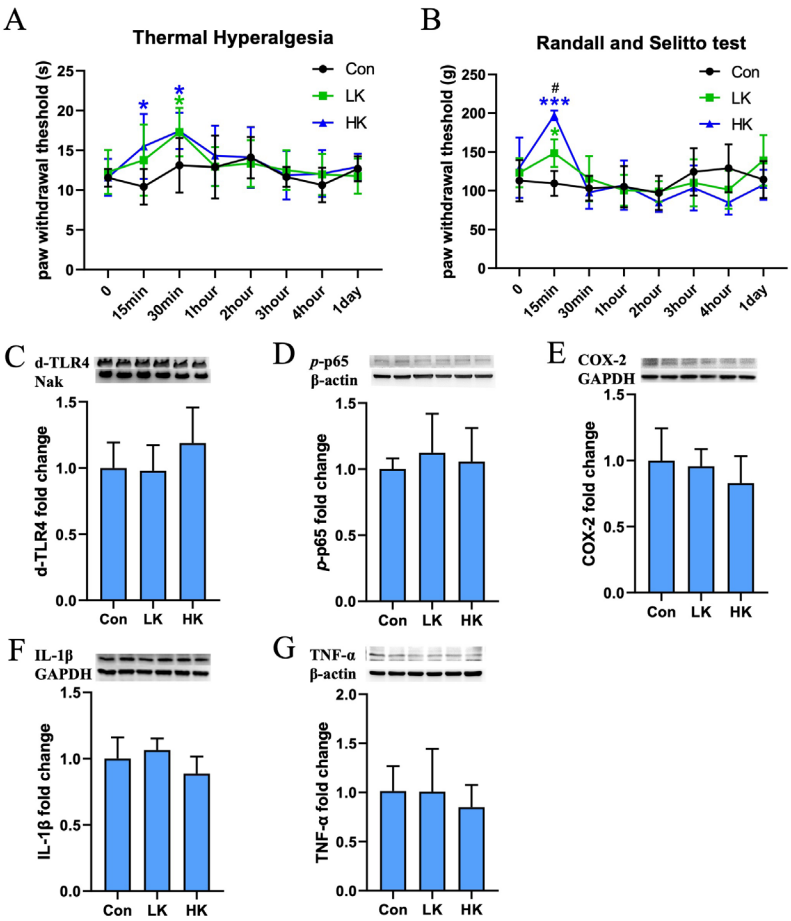

Figure 2. A single dose of ketamine had no long-term effects on naive rats. Ketamine 5 mg/mL or 10 mg/mL was injected into rats intraperitoneally. Thermal hyperalgesia **(A)** and allodynia **(B)** were determined from 15 min to 1 d after the injection (n = 6~8). The level of expression of d-TLR4 (n = 6) **(C)**, p-p65 (n = 6) **(D)**, COX-2 (n = 6) **(E)**, IL-1β (n = 6) **(F)**, and TNF-α (n = 6) **(G)** on day 1 after the ketamine injection was determined using Western Blotting. *$p < 0.05$, ***$p < 0.001$ *vs*. control group; $^{\#}p < 0.05$ *vs*. LK group. Data are expressed as the mean ± SD.

However, on days 1-7, the Fen group had a smaller AUC compared to the control group ($p < 0.001$) while the LKF ($p < 0.001$) and HKF ($p < 0.001$) groups had a higher AUC compared to the Fen group. The groups did not differ from the control group at the same time (Figure 3C).

The Randall-Selitto test was conducted to measure the mechanical pain threshold and yielded the same results as the Hargreaves test. The mechanical nociceptive threshold increased in the Fen group until 2 h (1 h: $p < 0.001$ and 2 h: $p < 0.001$) and in the HKF group until 3 h after drug injection (1 h: $p < 0.001$, 2 h: $p < 0.001$ and 3 h: $p = 0.0226$). However, the analgesic effect only lasted 2 h in the LKF group (1 h: $p < 0.001$ and $p = 0.0033$). The Fen group displayed a lower mechanical nociceptive threshold compared to the control group on day 1 to day 6 (day 1: $p = 0.0146$, day 2: $p = 0.0464$, day 3: $p = 0.003$, and day 6: $p = 0.0224$). The HKF (day 1: $p = 0.0439$ and day 4: $p = 0.0372$) group displayed a significant analgesic effect on day 1 and day 4 compared to the Fen group and did not differ from the control group (Figure 3D). Further calculation of AUCs revealed that the Fen ($p < 0.001$), LKF ($p < 0.001$), and HKF ($p < 0.001$) groups had a greater AUC than the control group in the first 4 h after the injection of fentanyl (Figure 3E). In contrast, the AUC for the Fen group on days 1 to 7 was significantly smaller than that for the control ($p < 0.001$), LKF ($p < 0.001$), and HKF ($p < 0.001$) groups (Figure 3F), indicating that the fentanyl-induced mechanical nociceptive threshhold was eliminated by ketamine.

Experimental results revealed significant hyperalgesia 1 to 4 d after fentanyl injection, and the animals' pain threshold returned to normal on day 7. Interestingly, a single injection of ketamine ahead of fentanyl can relieve mechanical allodynia and thermal hyperalgesia.

3.2. A single injection of ketamine in advance prevents the activation of the d-TLR4/ NF-κB pathway induced by fentanyl

To study the molecular mechanism by which ketamine prevents hyperalgesia induced by fentanyl, related protein expression was detected using Western blotting. Results suggested that d-TLR4 ($p = 0.0158$) (Figure 4A), *p*-p65 ($p = 0.0202$) (Figure 4B), COX-2 ($p = 0.0136$) (Figure 4C), IL-1β ($p = 0.0364$) (Figure 4D), and TNF-α ($p = 0.0314$) (Figure 4F) significantly increased in the spinal cord 1 d after fentanyl administration. The increased levels of d-TLR4 (Figure 4A) COX-2 (Figure 4C), and TNF-α (Figure 4E) induced by fentanyl can be reduced by a low dose of ketamine (d-TLR4: $p = 0.0262$, COX-2: $p = 0.0391$ and TNF-α: $p = 0.0229$), while a high dose of ketamine can reduce the levels of d-TLR4 ($p = 0.0015$) (Figure 4A), *p*-p65 ($p = 0.0030$) (Figure 4B), COX-2 ($p < 0.001$), IL-1β ($p < 0.001$) (Figure 4D), and TNF-α ($p = 0.0177$) protein expression.

Prior to day 7, there were no significant differences in levels of d-TLR4 (Figure 4F), *p*-p65 (Figure 4G), COX-2 (Figure 4H), IL-1β (Figure 4I), and TNF-α (Figure 4J) between the Fen group and the control group. Importantly, low or high doses of ketamine can reduce the levels of d-TLR4 (low dose: $p = 0.0032$ and high dose: $p = 0.0067$) (Figure 4F), COX-2 (low dose: $p = 0.0488$ and high dose: $p = 0.0397$) (Figure 4H), IL-1β (low dose: $p = 0.018$ and high dose: $p = 0.0083$) (Figure 4I), and TNF-α (low dose: $p < 0.001$ and high dose: $p < 0.001$) (Figure 4J) to various degrees but they do not alter the expression of *p*-p65 7 d after fentanyl injection (Figure 4G).

Immunohistochemical staining revealed that TLR4 (Figure 5A), *p*-p65 (Figure 5B), COX-2 (Figure 5C), and IL-1β (Figure 5D) co-localized with NeuN but not Iba-1 nor GFAP, indicating that TLR4, *p*-p65, COX-2, and IL-1β were expressed in neurons but not microglia nor astrocytes in the spinal cord on day 1 after fentanyl injection.

To sum up, the TLR4/NF-κ signaling pathway was activated 1 d after fentanyl injection and returned to normal 7 d later, which was consistent with behavioral results. A point worth noting is that a single injection of high-dose ketamine in advance alleviates the activation of TLR4/NF-κB better than low-dose ketamine on day 1. In addition, the combination of ketamine with fentanyl can still inhibit the TLR4/NF-κB/COX2 pathway on day 7.

3.3. Fentanyl-induced hyperalgesia and TLR4/NF-κB activation are prevented by inhibition of TLR4 with TAK-242

Thus far, results revealed that fentanyl injection led to hyperalgesia and activation of TLR4/NF-κB in the spinal cord, but causal links between TLR4/NF-κB activation and the abnormal behaviors in rats treated with fentanyl have not been established. To investigate this, the TLR4 inhibitor TAK-242 was injected 15 min before the first injection of fentanyl. Results indicated that TAK-242 substantially prevented the hyperalgesia induced by fentanyl ($p = 0.042$) (Figure 6A). Following the behavior tests, levels of TLR4, *p*-p65, COX-2, IL-1β, and TNF-α were measured using Western blotting, which revealed that the overexpression of TLR4 ($p = 0.0371$) (Figure 6B), *p*-p65 ($p = 0.0037$) (Figure 6C), COX-2 ($p = 0.0146$) (Figure 6D), IL-1β ($p = 0.0126$) (Figure 6E), and TNF-α ($p = 0.0422$) (Figure 6F) induced by fentanyl in the spinal cord was completely inhibited by TAK-242. These findings suggest that TLR4/NF-κB signaling in the spinal cord is essential for fentanyl-induced hyperalgesia.

4. Discussion

The current study found that the behavioral effects of thermal hyperalgesia in 1-2 d and mechanical allodynia

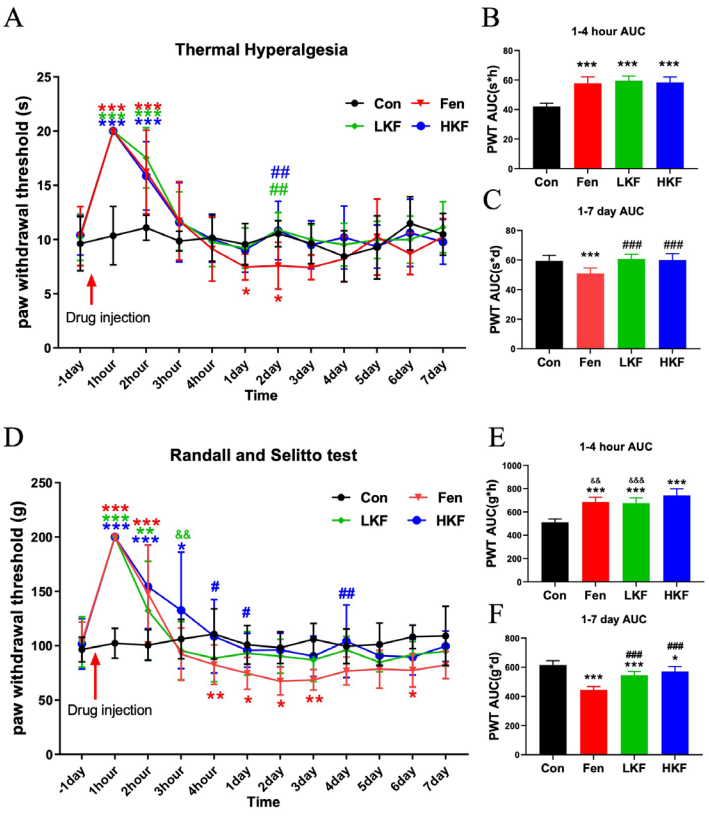

Figure 3. Pretreatment with ketamine reverses acute repeated fentanyl-induced hyperalgesia. Fentanyl 60 μg/kg was injected subcutaneously 4 times at 15-min intervals (Fen). Ketamine 5 mg/mL (LKF) or 10 mg/mL (HKF) was injected intraperitoneally 15 min before the fentanyl injection. Thermal hyperalgesia **(A)** and allodynia **(D)** were determined from 1 h to 7 d after drug injection. The AUC was calculated for hyperalgesia at 1-4 h **(B)**, hyperalgesia at 1-7 d **(C)**, allodynia at 1-4 h **(E)**, and allodynia at 1-7 d **(F)**. n = 9~12, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ *vs.* control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ *vs.* fentanyl group, $^{\&\&}p < 0.01$ *vs.* HKF group. Data are expressed as the mean ± SD.

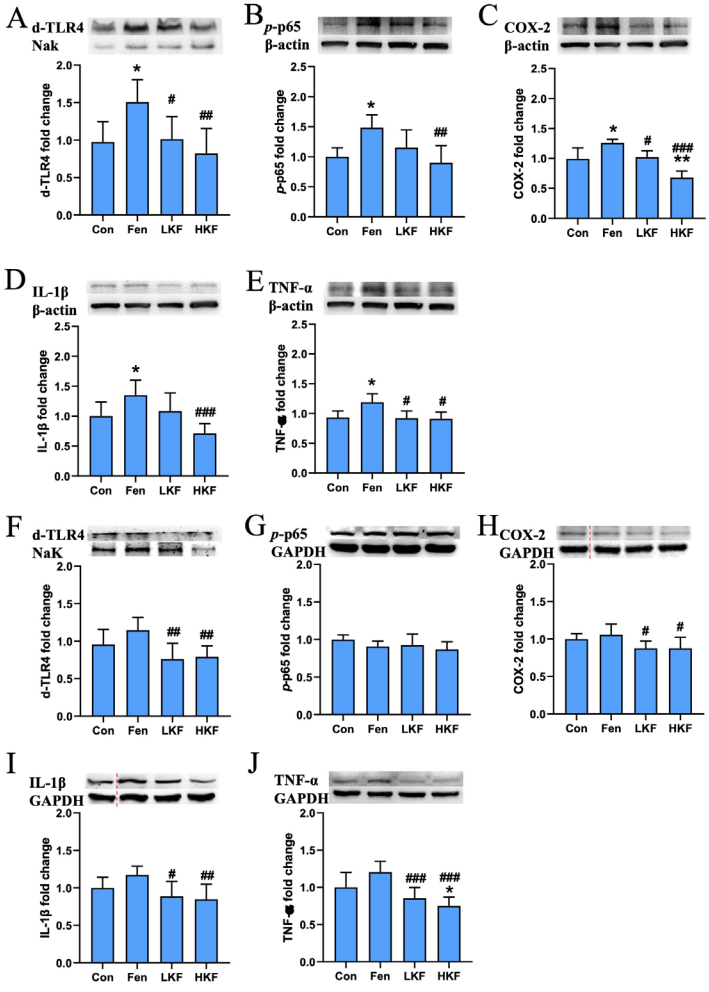

Figure 4. A single dose of ketamine can inhibit the high protein expression induced by acute repeated fentanyl injections on day 1 and its effect lasted for 7 d. One d after the drug injection following the drug injection protocol, the spinal cord was removed to determine the expression of TLR4 (n = 7) **(A)**, p-p65 (n = 6) **(B)**, COX-2 (n = 7) **(C)**, IL-1β (n = 8) **(D)**, and TNF-α (n = 6) **(E)** protein using Western Blotting. Fentanyl had no effect on the expression of TLR4 (n = 7) **(F)**, p-p65 (n = 4) **(G)**, COX-2 (n = 8) **(H)**, IL-1β (n = 8) **(I),** and TNF-α (n = 8) **(J)** protein expression on day 7. Ketamine decreased the expression of TLR4 **(F)**, COX-2 **(H)**, IL-1β **(I)**, and TNF-α **(J)** protein but not p-p65 **(G)** protein. *$p < 0.05$, **$p < 0.01$ *vs.* control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ *vs.* fentanyl group. Data are expressed as the mean ± SD.

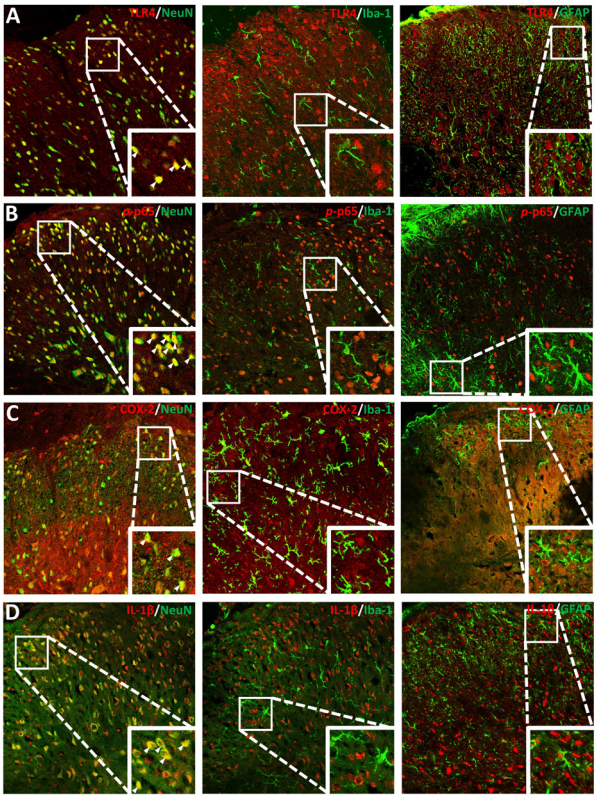

Figure 5. The localization of TLR4, *p*-p65, COX-2, and IL-1β in the spinal cord. One day after the fentanyl injection following the protocol, the spinal cord was removed to determine the localization of TLR4 **(A)**, *p*-p65 **(B)**, COX-2 **(C)**, and IL-1β **(D)**. TLR4, *p*-p65, COX-2, and IL-1β expressed in neurons (NeuN-positive cells) but not microglia (Iba-1-positive cells) nor astrocytes (GFAP-positive cells).

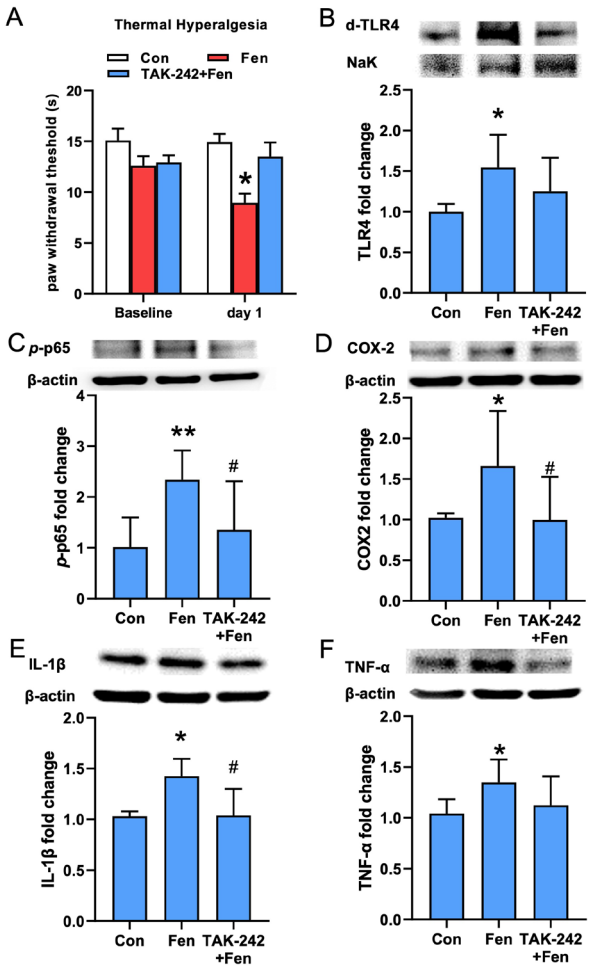

Figure 6. The TLR4 receptor contributed to fentanyl-induced-hyperalgesia. (A) The paw withdrawal threshold for rats before and one d after receiving fentanyl with/without TAK-242 (n = 7~9). The level of expression of d-TLR4 (n = 5~6). **(B)** The level of expression of *p*-p65 (n = 7~8) **(C)**, COX-2 (n = 8) **(D)**, IL-1β (n = 5) **(E)**, and TNF-α (n = 7~8) **(F)** on day 1 after the fentanyl and TAK-242 injection was determined using Western Blotting. *$p < 0.05$ and **$p < 0.01$ *vs.* control group; $^{\#}p < 0.05$ *vs.* fentanyl group. Data are expressed as the mean ± SD.

in 1-6 d induced by high doses of fentanyl in rats can be relieved by a single injection of ketamine in advance. This suggests that a single dose of ketamine had a significant effect at preventing fentanyl-induced hyperalgesia. In addition, the results of molecular experiments suggested that activation of the TLR4/NF-κB pathway was involved in fentanyl-induced hyperalgesia in spinal cord neurons and that ketamine reduced the activation of this pathway in a dose-dependent manner.

The current behavioral finding that a high dose of fentanyl can induce hyperalgesia is consistent with several clinical studies (*2, 4*) and animal studies (*11, 16*). However, other researchers (*3, 17*) have found that FIH lasted a few hours after injection. This difference in persistence over time may be due to the fentanyl dose. Interestingly, fentanyl-induced thermal hyperalgesia only lasts for 2 d while the mechanical nociceptive threshold can at least for up to 6 d. Some researcher have offered a few explanations for this phenomenon. One potential mechanism underlying mechanical allodynia is that Aβ fibers start to express some neuropeptides, which are usually expressed by small fibers (*18*). In some clinical studies, the ultra-lite components of heat-evoked potentials are described in the healthy after Aδ fiber blockade and C fiber sensitization (*19*). Different axon diameters and conduction velocities may lead to a different duration of allodynia and hyperalgesia in the current animal model.

The TRPV1 receptor (*20*), EphrinB receptor (*21*), and NMDA receptor (*9, 22-24*) are reported to be involved in OIH, which cannot comprehensively explain how ketamine reduces FIH. *p*-NF-κB in the spinal dorsal cord is reported to mediate morphine analgesic tolerance with increased TNF-α and IL-1β. The TLR4 inhibitor LPS-RS can alleviate morphine analgesic tolerance by reducing levels of *p*-NF-κB, TNF-α, and IL-1β (*25*). Hutchinson *et al.* found that the acute analgesic effects of morphine and oxycodone were significantly enhanced in TLR4 knockout rats (*26*). In a recent study, analgesic tolerance was prevented in TLR2 and TLR4 null mutant mice (*27*). In summary, the analgesic effect of opioids is enhanced

after TLR4 knockout, and the tolerance of opioids is alleviated. The current study found that TLR4/NF-κB mediated fentanyl-induced hyperalgesia. Therefore, the conjecture is that the enhanced analgesic effect of opioids after TLR4 knockout is due to reduced hyperalgesia. An important point to note is that, like the TLR4 inhibitor TAK-242, a single dose of ketamine can alleviate hyperalgesia and reduce the hyperactivation of TLR4/NF-κB induced by fentanyl. Studies have indicated that KC/GRO and TNF-α levels in male and female rats decreased 2 h after intravenous administration of ketamine, as did interleukin-6 (IL-6) levels in female rats and interleukin-10 (IL-10) levels in male rats (*28*). Low-dose ketamine inhibits neuroinflammation in PC12 cells by activating α7nAChR-mediated cholinergic anti-inflammatory pathways, such as the TLR4/MAPK/NF-κB signaling pathway (*29*), which suggests that ketamine may inhibit TLR4 expression in the same way.

The results from Figure 6 indicate that TLR4 may be one of the upstream proteins of COX-2, IL-1β, and TNF-α in fentanyl-induced-hyperalgesia. Bai *et al*. indicated that TLR4 was involved in morphine withdrawal-induced pain hypersensitivity by activating NF-κB, IL-1β, and TNF-α (*25*). In other pain models, such as arthritis hyperalgesia (*30*) and intraplantar-injected ceramide-induced-hyperalgesia (*31*), the inhibition of NF-κB can relieve hyperalgesia and decrease COX-2 expression. Moreover, morphine-3-glucoside (M3G), a morphine metabolite, can up-regulate TNF-α and COX2 mRNA expression and release prostaglandin E2 (PGE2) by activating the TLR4/NF-κB signaling pathway in central nervous system endothelial cells of rats (*32*). Interestingly, the current study found that repeated subcutaneous injections of fentanyl resulted in the overexpression of COX2, TNF-α, and IL-1β and that this can be inhibited by a single dose of ketamine or TAK-242. According to the aforementioned findings and the current findings, ketamine may relieve FIH by up-regulating inflammatory factors *via* the TLR4/NF-κB pathway.

Neurons mediate pain and densely innervate peripheral tissues through their nociceptors. A study has revealed that spinally projecting neurons were activated in the periaqueductal grey of adult mice after opioid injection (*33*). Ketamine is reported to act on neurons to reduce allodynia after injection of complete Freund's adjuvant (CFA) (*34*). Other researchers have even stated that the neurophysiological response of medullary pain-control neurons following chronic treatment with opioids is modulated by ketamine (*35*). Interestingly, the current study indicated that TLR4, NF-κB, COX-2, and IL-1β were co-localized with neurons, rather than astrocytes or microglia. Numerous studies have revealed that NF-κB is activated in neurons of the hippocampus, spinal cord, and dorsal root ganglion in models of chemotherapy-induced pain, while TNF-α and IL-1β are upregulated in neurons (*36, 37*). In addition, in ovariectomized and aged mice with chronic pain, TNF and Il-1β mRNAs were expressed in the neurons of DRGs and the spinal dorsal horn according to fluorescent in situ hybridization (FISH) (*38*). In a review, Roeckel *et al*. mentioned that neurons and glial cells activated by morphine will lead to OIH by producing cytokines such as IL-1β or TNF-α involved in LTP (*39*). Overall, these findings demonstrate that neurons in the central and peripheral nervous systems can modulate the production of proinflammatory cytokines, which may activate glial cells under pathological conditions. Neurons and glial cells interact with proinflammatory cytokines to produce continuous neuroinflammation and eventually lead to neuronal disease. The current study did not explore why TLR4, NF-κB, COX-2, and IL-1β are not expressed in glial cells 1 d after the fentanyl injection, so further research is needed to clarify this point. The neuron mechanisms found in the current study may have direct significance to clinical treatment of OIH.

In conclusion, results revealed that pretreatment with a single dose of ketamine relieved acute repeated FIH and increased levels of TLR4, NF-κB, COX-2, IL-1β, and TNF-α protein in spinal neurons. These results indicate an important role of the spinal neuron TLR4/NF-κB pathway in the manner in which ketamine relieves OIH. Preoperative or intraoperative administration of a single dose of ketamine may have a benefit on OIH and may improve the treatment of acute postoperative pain and even chronic post-surgical pain. Importantly, an early blockade of inflammation in spinal cord neurons rather than glial cells may interrupt the development of neuroinflammation, which may further prevent the activation of astrocytes and microglia and ultimately inhibit OIH initiated by neuroinflammation.

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