The Role of Toll-Like Receptor 4 Mediates Microglial Activation during Remifentanil-Induced Hyperalgesia in Rats

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Abstract

Background: Opioids can induce a state of nociceptive sensitization, also known as opioid-induced hyperalgesia. Nevertheless, the exact mechanism is still unclear. The following study investigates the role of Toll-like receptor 4 (TLR4) in the microglia activation during remifentanil—induced hyperalgesia in rats' model of incisional pain. Methods: Mechanical allodynia induced by remifentanil was established in adult male Sprague–Dawley rats with incisional pain. Paw withdrawal threshold (PWT) and paw withdrawal thermal latency (PWTL) were performed to evaluate mechanical and thermal hyperalgesia. The 32-G catheter intrathecal placement was used to deliver a specific TLR4 antagonist (LPS-RS).Western blot analysis was performed to measure the expression of the TLR4 and Iba-1, while Immunofluorescence staining was used to investigate the cell type and cell activation. Results Incisionalpain-remifentanil decreased the PWT and PWTL, upregulated the expression of TLR4 and microglia activation in the spinal cord. On the contrary, the intrathecal delivery of LPS-RS at the dose of 25 μg significantly decreased mechanical allodynia and prevented the upregulation of TLR4 induced by incisional pain-remifentanil Conclusion: These findings suggest that TLR4 signaling pathway has an important role in incisional pain-remifentanil hyperalgesia, and that it could serve as the therapeutic target for persistent postsurgical pain

Background

Postsurgical pain is a common complication following surgical operation that is observed in both adults and children [1, 2]. Clinical evidence has suggested that opioids can generate and strengthen postoperative pain sensitization, known as opioid-induced hyperalgesia (OIH) [3-6]. The most common side effects of opioids are respiratory depression and bradycardia, although very little is known about OIH. Hyperalgesia was defined as increased pain induced by a stimulus that usually causes pain by the International Association for the Study of pain[7]. Among different opioids, remifentanil provides the shortest context-sensitive half-time and final elimination half-life after 3 hours of infusion [8], and therefore, the use of remifentanil at high doses during surgery does not cause respiratory depression or delayed awakening. Clinically, it is commonly used for induction and maintenance of anesthesia. Nevertheless, recently, high doses of remifentanil infusion during surgery may increase postoperative pain and morphine demand[9][10]. Also, remifentanil-induced hyperalgesia caused by its faster and more frequent characteristic than that induced by other opioids, has become a focus problem[11][12][13].

Thus far, it has been proposed numerous opioid-receptor-dependent neuronal mechanisms of OIH[14]. In addition to activating classical opioid receptors, previous studies have shown that morphine activates toll-like receptor-4 (TLR4) on glia, triggering proinflammatory mediator release, which in turn activates a serious of cascade events that enhance nociception[15]. While neuronal morphine actions are analgesic, concurrent production of neuroexcitatory substances by glial cells (e.g. astrocytes and microglia) counteracts the analgesia, and eventually increases pain. Glial cells play a vital role in various physiological and pathological processes. Microglia cells, which are a subpopulation of glial cells, are currently recognized as a key role in the development of OIH [16][17]. Toll-like receptors (TLRs) have an important role in host defense during pathogen infection by linking and regulating specific and nonspecific immune responses[18-20]. TLR4 is a member of the TLRs family. It has been mainly expressed by the

microglia and is a transmembrane receptor protein with extracellular leucine-rich repeat domains and a cytoplasmic signaling domain[21-24].Moreover, morphine[25], oxycodone[26]and codeine[17]have shown the ability to induce the activation of TLR4 in glial cells, while its activation by remifentanil remains unexplored. The following study investigates the role of TLR4 in the microglia activation during remifentanil—induced hyperalgesia in rats' model of incisional pain.

Methods

Animals

Adult male Sprague-Dawley rats weighing 240 to 260 g were taken from Central Animal center, Anhui Medical University, China. Every four animals are raised in groups and adapted to the housing environment for one week. The ambient temperature is 22 ± 1 ºC, the relative humidity is 50 ± 10%, and the light and dark period is 12 / 12 hr. The protocol had been approved by the Ethical Committee of Anhui Medical University. In addition, all animal studies (including the rat euthanasia procedure) were done in compliance with the regulations and guidelines of Anhui Medical University institutional animal care and conducted according to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the Institutional Animal Care and Use Committee (IACUC) guidelines.

Surgery procedure

The plantar incision was performed according the method described by Brennan et al[27]. Rats were anesthetized by nasal mask with sevoflurane (induction, 3%; surgery, 1%). The plantar surface of the right hind paw was disinfected with 5 % povidone-iodine solution and the animal foot through a hole in a sterile drape. A 1cm longitudinal incision through the skin and fascia starts at 0.5 cm from the margin of the heel, extending to the toes of the right hind paw. Using tweezers to raise the plantar muscle and cut it longitudinally to keep the muscle source and insertion intact. After stopping bleeding with gentle pressure, seal the skin with two 5 - 0 nylon mattress sutures. The wound was covered with erythromycin ointment. The incision was checked daily to exclude animals with signs of infection or dehiscence from the study.

Drug administration and Behaviors Testing

Remifentanil(0.04 mg/kg, 0.4 ml, 0.8ml/h, Ren Fu Co, Hubei, China, 6171003). Sevoflurane(Heng Rui Co, China, 17092731). Remifentanil was dissolved in saline (NaCl 0.9%; 0.4 ml) and then subcutaneously injected using an apparatus pump during consecutive 30-min.

For intrathecal drug administration, 32-G intrathecal catheters were inserted through the atlanto-occipital membrane into the lumbar enlargement under anesthesia. Excluding animals from the study that failed to show paralysis of the hind limbs by lidocaine(2%,5ml).
Lipopolysaccharide, an inhibitor of TLR 4 from the photosynthetic bacteria *Rhodobacter sphaeroids* (LPS-RS, 25ug per 10uL; InvivoGen, San Diego, CA) were administrated intrathecally 30 min before Plantar Incision surgery.

To evaluate mechanical hyperalgesia, paw withdrawal threshold (PWT) was assessed by Von Frey filaments (Cat.38450, Ugo Basile, Varese, Italy). Each animal was placed alone in a cage (20 cm×20 cm ×20 cm) with a wire mesh grid floor in a quiet room. To avoid disruption of the wound, Von Frey filaments were inserted vertically to the hind paw plantar surface adjacent to the incision. Each rat was tested three times at intervals of 5 minutes.

Paw withdrawal thermal latency (PWTL) measured with test equipment (BME410C, Institute of Biological Medicine, Academy of Medical Science, China) to evaluate thermal hyperalgesia. A transparent plastic chamber (22 cm × 12 cm × 12 cm) with a glass floor (2 mm thick) was used to place rats. The plantar surface adjacent to the wound of right hind paw was focused by a radiant heat source under the glass floor. The time from onset of radiant heat to withdrawal of the rat hind paw was defined as the withdrawal latency to the heat stimulation. In order to prevent tissue damage, a cut off time of 25s was established. Each rat was tested three times at intervals of 5 minutes. Thermal latency was defined as the mean of three responses.

Experimental Protocol and Grouping

Rats were randomly divided into five groups (num=8/group): group N, rats underwent a sham operation and were administered with same volume of saline ; group I, rats underwent a incision operation and received saline; group R, received a subcutaneous injection of remifentanil but did not undergo surgery; group R+I, rats underwent a incision operation and received subcutaneous injection of remifentanil; group L, received an intrathecal injection of LPS-RS 30 min before plantar incision in remifentanil-treated rats. Incision surgery and drug injection were performed simultaneously.

The same investigator performed all the experiments. For von Frey and plantar tests, baseline responses were obtained one day before surgery after animals became familiar with the special assessment conditions without nociceptive stimulation. According to the above protocol, the experiments were performed 1 day later. PWT and PWTL tests were conducted at 2, 6, 24 and 48 hours postoperative. After the behavioral testing (48h after operation), immunofluorescence staining and Western blot analysis specimens were collected.

Immunofluorescence

Rats transcardially perfusion with saline, followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH = 7.4) While under deep anesthesia (5% sevoflurane). The lumbar segments (L4-6) of the spinal cord were then dissected and fixed in the same fixative for 3 h and then replaced with 30% sucrose overnight. Cryostat sections(10 mm) were cut and incubated with 20% normal goat serum at room temperature for 30 minutes and then diluted with primary antibody anti-Iba-1(microglia marker 1:500; Abcam, Cambridge, UK, ab5076)) for 24 h at 4°C. After incubation at 4 °C for one night, the sections were incubated in Cy3-conjugated and FITC-conjugated secondary antibodies for 1h at room temperature. A fluorescence microscope (Leica, Frankfurt, Germany) was used to examine the section and images were captured using a Leica DFC350 FX camera. Each group included four rats for

immunofluorescence quantification, and tissue sections of each animal were randomly selected for analysis.

Western Blotting

The lumbar segments (L4-6) of the spinal cord were removed rapidly and snap frozen in liquid nitrogen while under deep anesthesia (5%sevoflurane). Tissue samples were homogenized in lysisbuffer solution.The supernatant was obtained by centrifugation for 15 min, at 4°C for 13000 rpm. Bradford method, a detergent-compatible protein assay with a bovine serum albumin as standard is used to determine the protein concentration. Samples (80μg) were separated on SDS-PAGE (10%) and transferred to PVDF membrane. The filter membranes were blocked with 5% nonfat milk for 1h at room temperature and incubated with rabbit antibody against TLR4 (1:500; Abcam, Cambridge, UK, ab13556), iba1(1:2000; Abcam, Cambridge, UK, ab5076) at 4°C overnight. TBST buffer was used to wash the membrane and then the membrane was incubated for 1h at room temperature with secondary antibody conjugated with horseradish peroxidase, observed in ECL solution for 1 minute, and then exposed for 1-10 minutes. The membrane was reprobed with antibody against beta actin (1:1000, SC-47778, Santa Cruz, USA) to verify the loading and blotting of equal amount of proteins. Densitometry with a computer-assisted imaging analysis system (ImageJ; NIH, Bethesda, MD) was used to analyze the band intensities.

Statistical Analysis

All data were analyzed with SPSS 16.0. Statistical analyses of behavioral testing data were performed using two-way analysis with repeated measures. The results of Western blot and immunofluorescence were analyzed by one-way ANOVA. Data of all experiments were expressed as mean ± SD. $P< 0.05$ was considered statistically significant.

Result

The effects of intraoperative remifentanil infusion on PWT and PWTL during the postoperative period

Sevoflurane induction and subcutaneous injection of saline for 30 min
had no significant effect on PWT and PWTL in rats without operation compared to baseline (24h before operation) ($P >0.05$). However, in other groups, the nociceptive threshold decreased from 2h to 48h after operation. The PWT and PWTL of the rats with remifentanil administration at 2, 6, 24 and 48 hours postoperative were significantly lower (all $P <0.01$) compared to group I and R. Nevertheless, preoperative intrathecal injection of LPS-RS could significantly reduced mechanical pain sensitivity ($P < 0.01$) and thermal allodynia ($P <0.01$) caused by remifentanil infusion during surgery (**Figure 1 and Figure 2**).

Immunofluorescence staining of microglia activation in spinal cord

Immunofluorescence staining was performed to localize and assess microglia activation in the spinal cord during the maintenance of hyperalgesia induced by intraoperative remifentanil infusion. The phosphorylation Iba-1 was located in the spinal cord was showed by typical photomicrographs (**Figure 3A**).

The mean optical density of Iba-1 in the spinal cord was summarized in **Figure 3B**. The microglia activation in the spinal cord was weak in rats receiving sevoflurane and saline without surgery, However there was a significant increase in the group I, R, R+I, and L compared to group N (all $P < 0.01$). Moreover, Intraoperative Remifentanil infusion significantly enhanced microglia activation in the spinal cord ($P < 0.01$). Conversely, pretreatment with LPS-RS could reduce the microglia activation in spinal cord caused by intraoperative infusion of remifentanil ($P <0.01$).

Western Blot Analysis

Western blot was used to quantify the expression of Iba-1 and TLR4 in the spinal cord during the maintenance of hyperalgesia induced by remifentanil. Intraoperative Remifentanil infusion increased the level of Iba-1 and TLR4 in the spinal cord compared to the rats who received sevoflurane and saline without surgery and intraoperative saline infusion ($P< 0.01$). The LPS-RS pretreatment reduced the higher level of TLR4 in spinal cord caused by Intraoperative remifentanil ($P < 0.01$) (**Figure 4**).

Discussion

Opioid-induced hyperalgesia has been widely documented in several models of acute and chronic pain. In our study, we used a rat model of postoperative pain to investigate whether the intraoperative administration of opioids would alter the nociceptive response to surgery and related mechanisms. We found that intraoperative remifentanil infusion decreased nociceptive thresholds and upregulated activation of microglia and the expression of TLR4 in spinal cord, while the inhibition of TLR4 signaling by LPS-RS decreased the mechanical allodynia induced intraoperative remifentanil infusion.

Microglia are the first and most important defense line of the central nervous system (CNS) .Clinical researches have shown that activated microglia plays an important role in the pathogenesis of neurodegenerative diseases, Like Parkinson's disease and multiple sclerosis.TLR4 widely expressed in the CNS. Accumulating studies show that the activation of TLR4 is closely related to the development and maintenance of pathological pain[29-30]. And it also has shown to play a key role in neuroinflammation in central nervous system trauma diseases and several neurodegenerative diseases[31-34]. The present study showed that intraoperative remifentanil infusion upregulated TLR4 expression in spinal cord, while the inhibition of TLR4 signaling by LPS-RS significantly reduced the incisional pain-remifentanil induced mechanical allodynia and TLR4 expression. This indicates that TLR4 plays an important role in OIH.

In this model, sevoflurane inhalation and continuous infusion of remifentanil was imitated the administration of general anesthesia in humans. And the remifentanil (0.04 mg/kg) dose was selected according to the existing reference which shows that a loss of righting reflex after the infusion of remifentanil in rats is predictive of clinical anesthesia[35]. Celeri *et al*. have reported that remifentanil, that was administered to rats with incisional pain to simulate hyperalgesia, could induce a strong hyperalgesia 2 h after operation, while it can reach a peak within (24 -48) h[36]. Accordingly, the time points observed in this study were 2, 6, 12, 24 and 48h postoperative, and the time of taking materials was 48h after operation

with the strongest hyperalgesia. The major shortcoming of this study is that it did not include TLR4 Immunofluorescence staining to further verify the TLR4 expression on the microglia.

Conclusion

In the present study we demonstrated the involvement of TLR4 signaling pathway in spinal cord in the mechanical allodynia induced by incisional pain-remifentanil. We found that intraoperative remifentanil infusion induced the upsurge of TLR4 and microglia activation in spinal cord, while the inhibition of TLR4 signaling using LPS-RS reduced mechanical allodynia and prevented the upregulation of TLR4 and microglia activation induced by incisional pain-remifentanil. Our findings revealed that TLR4 signaling pathway has an important role in incisional pain-remifentanil hyperalgesia, and that it could serve as the therapeutic target for persistent postsurgical pain.

Declarations

Ethics approval and consent to participate

We confirm that this protocol had been approved by the Ethical Committee of Anhui Medical University. In addition, all animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Anhui Medical University institutional animal care and conducted according to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the Institutional Animal Care and Use Committee (IACUC) guidelines.

Consent for publication

Not Applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article [and its supplementary information files]

Competing interests

The authors declare that they have no competing interests. We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agree with submission to the BMC Anesthesiology. We have read and have abided by the statement of ethical standards for manuscripts submitted to the BMC Anesthesiology.

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Authors' contributions

Conceived and designed the experiments: WX CXX. Performed the experiments CXX Analyzed the data CXX WX. Contributed reagents/materials/analysis tools WX Contributed to the writing of the manuscript: WX CXX

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Tables

PWT						PWTL				
N										
	baseline	2h	6h	24h	48h	baseline	2h	6h	24h	48h
1	22.7	22.4	22.1	22.4	22.9	16.9	16.6	16.8	16.6	16.6
2	22.6	22.5	22.2	23	22.4	16.8	16.8	16.7	16.5	16.6
3	21.9	22.3	22.4	22.5	22.1	16.4	16.6	16.8	16.8	16.7
4	22.3	22.3	22.6	22.3	21.7	16.9	16.9	16.6	16.7	16.5
5	23	23	22.9	22.5	22.7	16.7	16.7	16.5	16.4	16.7
6	22.8	22.6	22.5	22.9	23	16.5	16.5	16.7	16.7	16.8
7	22.8	22.5	22.9	22.3	22.5	16.9	16.8	16.9	16.8	16.4
8	22.9	23	22.5	22.4	22.3	17	16.7	16.8	16.6	16.9
I						16.7	12.4	10.8	8.9	8.3
1	22.5	13.9	13	11.3	11.1	16.4	12.3	10.8	8.8	8.3
2	22.4	13.8	12.9	11.2	11.3	16.8	12.5	10.7	8.7	8.4
3	22.3	13.8	13.1	11.3	11.4	16.9	12.3	10.8	8.9	8.2
4	22.6	13.7	13.2	11	11.1	16.5	12.6	10.7	8.6	8.4
5	23	14	12.9	11.4	10.9	16.8	12.4	10.9	8.7	8.5
6	22.5	13.6	13.1	11.3	11.2	16.7	12.5	10.6	8.5	8.3
7	22.2	13.6	13.1	11.2	11.3	16.6	12.4	10.8	8.9	8.4
8	22.3	13.5	13	11.3	11.2					
R						16.8	12.3	10.7	8.9	8.7
1	22.6	13.9	13.1	11.3	11.2	16.6	12.3	10.7	8.9	8.7
2	22.5	13.8	13.2	11.5	11.3	16.9	12.4	10.6	8.8	8.6
3	22.4	13.9	12.9	11.2	11.4	16.7	12.6	10.5	8.6	8.8
4	22.6	14	13.4	11.6	11.6	16.5	12.4	10.6	8.8	8.6
5	22.4	13.4	13.3	11.2	11.1	16.8	12.6	10.7	8.7	8.7
6	22.3	13.7	13.1	11	10.9	16.7	12.5	10.7	8.9	8.5
7	23	13.8	13.6	11.4	11	16.8	12.6	10.6	8.9	8.5
8	22.9	13.7	13.4	11.4	11.3					

RI						16.9	11.3	8.2	6.2	5.8
1	22.4	13	11.7	8.8	8.5	16.5	11.2	8.2	6.2	5.6
2	22.5	13	11.8	8.7	8.4	16.8	11.2	8.3	6.4	5.8
3	22.6	13.4	12	9	8.7	16.7	11.5	8.5	6.1	5.7
4	22.4	13.2	11.7	8.6	8.5	16.4	11.3	8.3	6.3	5.6
5	22.3	12.9	11.6	8.7	8.3	16.6	11.4	8.4	6.2	5.7
6	22.4	13.2	11.9	8.5	8.6	16.7	11.3	8.6	6.2	5.5
7	22.5	13.1	12	8.6	8.5	16.9	11.4	8.4	6.1	5.8
8	22.6	13.5	11.7	8.7	8.7					
L						16.8	12	9.9	7.9	7
1	22.5	13.7	12.8	10.4	9.9	16.4	12.1	10.1	8.1	7
2	22.6	13.7	12.5	10.5	10	16.9	11.9	10.2	8.1	7.1
3	22.4	13.6	12.6	10.3	10	16.7	12.1	10.2	8	7.1
4	22.3	13.7	12.9	10.3	9.8	16.5	12.1	10.4	7.9	7.3
5	22.4	13.6	12.7	10.2	9.9	16.8	12.3	10.2	8	7.2
6	22.5	13.5	12.7	10.2	10	16.6	12	10.3	8.2	7.3
7	22.3	13.4	12.4	10.3	10	16.8	12	10.3	8.3	7.2
8	22.3	13.5	12.9	10.4	9.8					

Figures

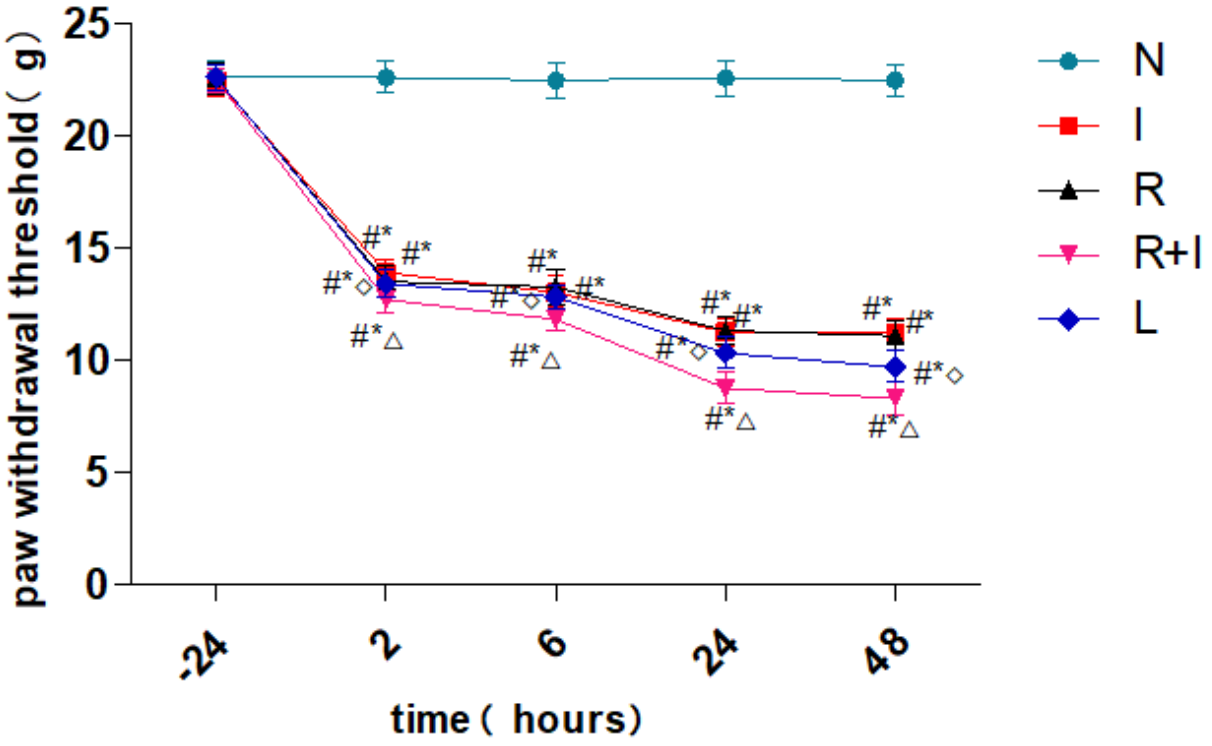

Figure 1

Effects of remifentanil on PWT during the postoperative period and the intervention of LPS-RS. LPS-RS (25ug per 10 uL) intrathecal injected 30 min before surgery. Under sevoflurane anesthesia, remifentanil (0.04 mg/kg, 0.4 ml) or saline was subcutaneously infused in the absence or presence of the right hind paw incision during a period of 30 min. PWT was evaluated at 24 h before (baseline)and at 2 h, 6 h, 24 h and 48 h after surgery. Number of rats per group was eight. Data are expressed as means ± SD. *P < 0.01 vs baseline, # P < 0.01 vs group N, △P < 0.01 vs group I or R, ◇P < 0.01 vs group R+I.

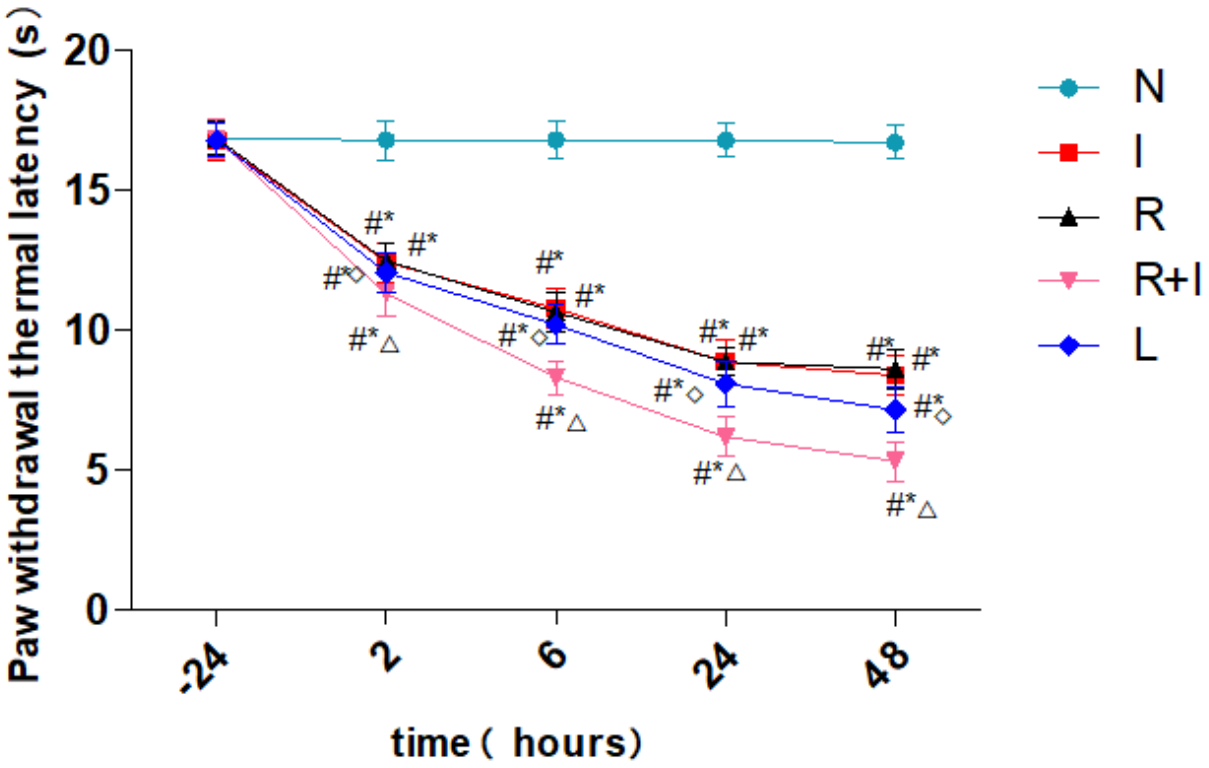

Figure 2

Effects of remifentanil on PWTL during the postoperative period and the intervention of LPS-RS LPS-RS (25ug per 10 uL) intrathecal injected 30 min before surgery. Under sevoflurane anesthesia, remifentanil (0.04 mg/kg, 0.4 ml) or saline was subcutaneously infused in the absence or presence of the right hind paw incision during a period of 30 min. PWTL was evaluated at 24 h before (baseline)and at 2 h, 6 h, 24 h and 48 h after surgery. Number of rats per group was eight. Data are expressed as means ± SD. *P < 0.01 vs baseline, # P < 0.01 vs group N, △P < 0.01 vs group I or R, P < 0.01 vs group R+I.

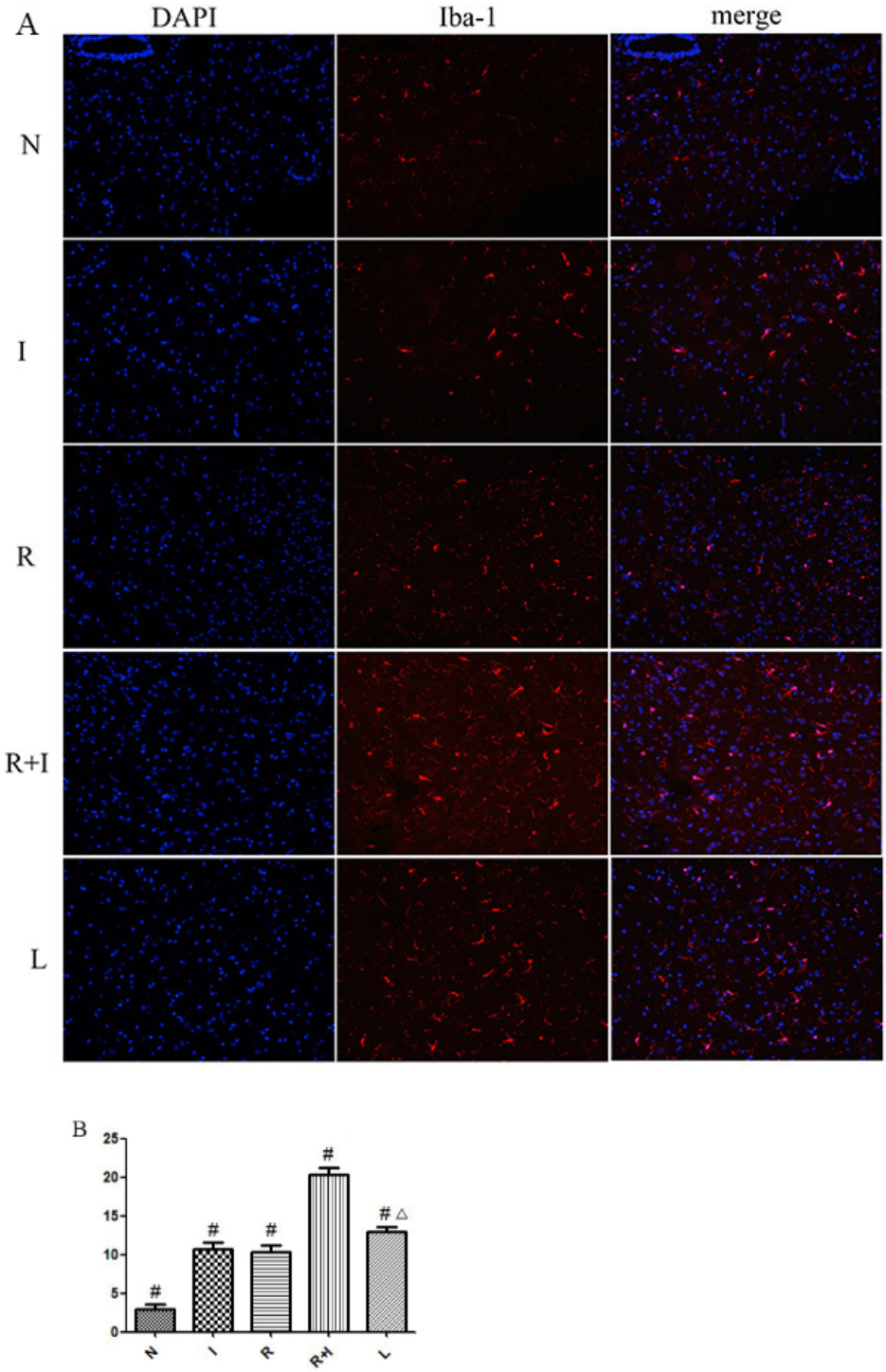

Figure 3

Immunofluorescence staining of microglia marker Iba-1 in groups. (IF×200) When compared with group N, group I, R, R+I increased the level of Iba-1 in the spinal cord (P <0.01). The expression of iba-1 in rats receiving intraoperative remifentanil infusion was significantly upregulated when compared with rats receiving intraoperative saline infusion (P< 0.01). Pretreatment of LPS-RS decreased the higher level of Iba-

1 in the spinal cord caused by remifentanil (P < 0.01).Data are expressed as means ± SD. # P < 0.01 vs group N, *P < 0.01 vs group R or I, △P < 0.01 vs group R+I.

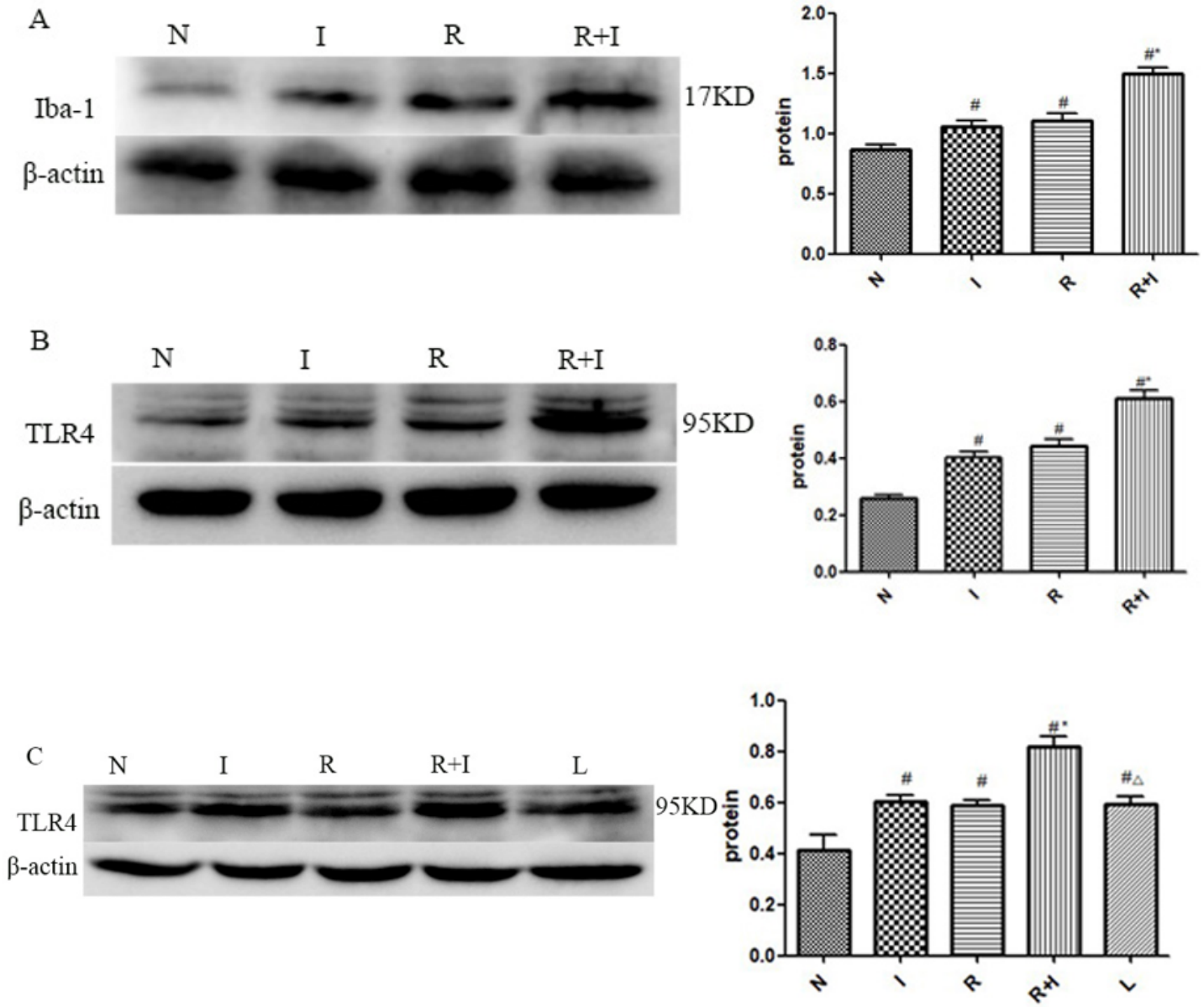

Figure 4

The expression level of Toll-like receptor 4 (TLR4) or Iba-1 in each group. (A) When compared with group N, group I, R, R+I increased the level of Iba-1 in the spinal cord (P <0.01). The expression of Iba-1 in rats receiving intraoperative remifentanil infusion was significantly upregulated when compared with rats receiving intraoperative saline infusion (P< 0.01). (B) When compared with group N, group, R, R+I increased the level of TLR4 in the spinal cord (P <0.01). The expression of TLR4 in rats receiving intraoperative remifentanil infusion was significantly upregulated when compared with rats receiving intraoperative saline infusion (P< 0.01). (C) Pretreatment of LPS-RS decreased the higher level of TLR4 in spinal cord caused by remifentanil (P < 0.01) .Data are expressed as means ± SD.# P < 0.01 vs group N, *P < 0.01 vs group R or I, △P < 0.01 vs group R+I.

Supplementary Files

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- supplement1.docx