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The Molecular Mechanisms Associated with the Effects of Propofol in a Rat Model of Pain Due to Inflammation Following Injection with Complete Freund's Adjuvant

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Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEFG 1 **Shanshan Tan**
BCD 2 **He Liu**
BD 3 **Yuanzheng Wang**
ACEG 3 **Shanshan Zhu**

1 Department of Anesthesiology, Nanjing Hospital of Chinese Medicine Affiliated to Nanjing University of Chinese Medicine, Nanjing, Jiangsu, P.R. China
2 Department of Anesthesiology, The Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu, P.R. China
3 Department of Anesthesiology, Xuzhou Cancer Hospital, Xuzhou, Jiangsu, P.R. China

Corresponding Author: Shanshan Zhu, e-mail: zhuss190601@163.com
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Background: This study aimed to investigate the molecular mechanisms associated with the effects of propofol in a rat model of pain due to inflammation following subcutaneous injection with complete Freund's adjuvant (CFA).

Material/Methods: Sprague-Dawley rats were injected subcutaneously in the paw with CFA. Propofol or saline was administered by tail vein injection. After CFA treatment for 0 hours, 4 hours, 1 day, 4 days, 7 days, and 14 days, the behavior of the rats was assessed. An enzyme-linked immunosorbent assay (ELISA) measured serum levels of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6. Western blot and the quantitative reverse-transcription polymerase chain reaction (qRT-PCR) were used to detect levels of p38MAPK and NF-κB related mRNA and proteins, including p-p38, p38, p65, p-p65, NOD-like receptor family protein 3 (NLRP3), apoptosis-associated speck-like protein (ASC) and caspase-1 in rat spinal cord tissues.

Results: Injection of CFA significantly reduced the mechanical withdrawal threshold (MWT), thermal withdrawal latency (TWL), and frequency responses to cold stimulation. Propofol treatment significantly reduced serum levels of TNF-α, IL-1β, and IL-6. Protein expression levels of p-p38 and p-p65 were upregulated in the rat model, which were inhibited by propofol treatment. CFA injection increased the expression levels of NLRP3, ASC, and caspase-1 in the spinal cord tissues of rats, which were reduced by propofol treatment.

Conclusions: In a rat model of pain following subcutanous injection with CFA, propofol reduced CFA-induced pain and inhibited the inflammatory response through the p38MAPK-nuclear factor-κB (NF-κB) pathway and the NLRP3 inflammasome.

MeSH Keywords: **MAP Kinase Kinase Kinases • NF-kappa B • Pain • Propofol**

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Background

Chronic inflammation results in tissue injury and inflammatory and other mediators associated with inflammation may cause pain, which is an important clinical symptom that may be spontaneous or evoked, and may be associated with hyperalgesia [1,2]. Inflammatory pain hypersensitivity reflects early posttranslational molecular changes [3]. Clinically, the pain associated with inflammation affects patient quality of life [4,5]. Current medications for pain combined with inflammation include nonsteroidal anti-inflammatory drugs (NSAIDs) [6]. However, the long-term use of NSAIDs can be associated with serious gastrointestinal side effects, including bleeding [6]. Therefore, continued studies are needed to identify safe and effective pain medications.

Propofol is a commonly used intravenous anesthetic, which is frequently used for both anesthesia and analgesia [7]. Recently, propofol has been shown to be effective in treating conditions such as migraine and epilepsy, but there have been few studies on its effects in pain associated with inflammation [8–10]. Previously published studies have shown that propofol inhibited cyclic adenosine monophosphate (cAMP) production through the protein kinase C-dependent pathway, to reduce beta-adrenergic signal transduction in cardiomyocytes [11]. The invasion of pancreatic cancer cells was reduced and apoptosis was promoted following propofol treatment, which was mediated through the miR-21/SLUG signaling pathway [12]. Propofol has previously been shown to reduce the secretion of proinflammatory factors, inhibit the attachment, migration, and phagocytosis of neutrophils, and to inhibit the production of reactive oxygen species (ROS). Hsing et al. [13] demonstrated that propofol reduced the expression of the inflammatory, tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein 1 (MCP-1), and inhibited oxidative stress by increasing the expression of bone morphogenetic protein-7 (BMP-7). Also, propofol inhibited lipopolysaccharide (LPS)-induced inflammation in macrophages through regulation of the ROS/AKT/IKK-β/NF-κB signal pathway [14]. However, there have been few previous studies on the effects of propofol on pain associated with inflammation.

A rat model of pain associated with inflammation has previously been reported, which was established using subcutaneous injection with complete Freund's adjuvant (CFA) [15,16]. Therefore, this study aimed to investigate the molecular mechanisms associated with the effects of propofol in a rat model of pain due to inflammation following subcutaneous injection with CFA.

Material and Methods

Animals

Healthy male Sprague-Dawley rats (4–6 weeks old), and weighing 150–200 g, were obtained from the Experimental Animal Center of the Fourth Military Medical University (FMMU), China, and were maintained in a standard animal room with a 12-hour light and dark cycle with a standard temperature and were fed under standard conditions. All protocols of the animal experiments were strictly performed following the Institutional Animal Care and Use of Laboratory Animals by the National Institutes of Health. All experimental protocols were approved by the Animal Care and Use Committee of Xuzhou Medical University.

Establishment of the rat model and the study groups

The rat model of inflammatory pain was established using a subcutaneous injection of 100 µl of complete Freund's adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO, USA) into the plantar surface of the right hind paw of the Sprague-Dawley rats, as previously described [15].

Rats were randomly assigned into six groups (n=10 per group). In the Control group, the rats were injected with 100 µl of normal saline in the tail vein once every day for four consecutive days. The CFA group included Sprague-Dawley rats injected subcutaneously with 100 µl of CFA. The CFA+saline group were injected in the tail vein with the same volume of normal saline and propofol, and one hour later, the rats were injected with 100 µl of CFA. The CFA+propofol group were injected with 10, 20, and 40 mg/kg of propofol in the tail vein, and one hour later, the rats were treated with 100 µl of CFA. In the CFA+propofol group, tail vein injections of propofol were performed once every day for four consecutive days.

Behavioral tests

The rats underwent assessment using behavioral tests, which including evaluation of the mechanical withdrawal threshold (MWT), the thermal withdrawal latency (TWL), and the frequency responses to cold stimulation. Von Frey monofilaments (Stoelting Co., Wood Dale, IL, USA) were used to evaluate mechanical hyperalgesia. The back paw of the rats underwent increasing pressure, with each application lasting for 5–6 seconds and the minimum force that resulted in paw withdrawal was identified as the MWT. A radiant heater (BME-410A, Beijing, China) was placed under the plantar surface of the back paw for the thermal preference test, and the response of each back paw was evaluated in triplicate at intervals of 3 minutes. TWL was noted when the hind paws were removed from the thermal plate at 40-second intervals to avoid tissue damage.

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For the thermal preference test, a syringe connected to a thin polyethylene tube was applied to smear a drop of acetone to each back paw. The test was performed in triplicate at intervals of 5–10 minutes between each test. All the tests were performed at 0 h, 4 h, 1d, 4 d, 7 d, or 14 d after CFA injection.

Enzyme-linked immunosorbent assay (ELISA)

Four days after CFA injection, antibodies were used (Beyotime, Shanghai, China) to detect the serum levels of TNF-α (Cat no. PT519), IL-1β (Cat no. PI303) and IL-6 (Cat no. PI328) using an enzyme-linked immunosorbent assay (ELISA) kit (Beyotime, Shanghai, China), following the manufacturer's protocol. Each set of experiments was performed in triplicate.

Western blot

Four days after CFA injection, the rats were euthanized, and spinal cord tissues were removed and treated with radioimmunoprecipitation assay buffer (Beyotime Biotechnology, China) mixed with phenylmethyl sulfonyl fluoride (PMSF), and centrifuged to obtain the total protein. A bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA) was used to determine the protein concentration. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with 5% dried skimmed milk powder for 1 h at room temperature, and incubated overnight at 4°C with the primary antibodies. The primary antibodies used were to p-p38 (1: 1000) (Cat no. 4511; Abcam, Cambridge, MA, USA), p38 (1: 1000) (Cat no. 8690; Cell Signaling Technology, Danvers, MA, USA), p-p65 (1: 1000) (Cat no. 3033; Cell Signaling Technology, Danvers, MA, USA), p65 (1: 1000) (Cat no. 8242; Cell Signaling Technology, Danvers, MA, USA), NLRP3 (1: 1000) (Cat no. ab232401; Abcam, Cambridge, MA, USA), apoptosis-associated speck-like protein (ASC) (1: 1000) (Cat no. sc-271054; Abcam, Cambridge, MA, USA), caspase-1 (1: 1000) (Cat no. ab1872; Abcam, Cambridge, MA, USA), and GAPDH (1: 1000) (Cat no. 5174; Cell Signaling Technology, Danvers, MA, USA). After five washes in phosphate-buffered saline Tween-20 (PBST), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1: 1000) (Cat no. sc-516102: Santa Cruz Biotechnology Inc., Dallas, TX, USA) and (1: 2,999) (cat no. 7074) (Cell Signaling Technology, Danvers, MA, USA) for 1 h at 37°C. Finally, the protein bands were detected using enhanced chemiluminescence (ECL) reagent (Bio-Rad, Hercules, CA, USA).

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from spinal cord tissues of rats using an RNA-isolation kit (Invitrogen, Carlsbad, CA, USA), and complementary DNA (cDNA) was synthesized by using PrimeScript™ RT reagent Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The relative levels of NLRP3, ASC, and caspase-1 mRNA were quantified by using the Power SYBR Green Master mix (Vazyme, Piscataway, NJ, USA). Primers were provided by Sangon Biotech (Shanghai, China) and listed as follows:
GAPDH, forward: 5'-AAAATCAAGTGGGGCGATGC-3';
GAPDH, reverse: 5'-AGGAGGCATTGCTGATGATCT-3';
NLRP3, forward: 5'-GATCTTCGCTGCGATCAACAG-3';
NLRP3, reverse: 5'-CGTGCATTATCTGAACCCCAC-3';
ASC, forward: 5'-GCAATGTGCTGACTGAAGGA-3'
ASC, reverse: 5'-TGTTCCAGGTCTGTCACCAA-3';
Caspase-1, forward: 5'-GCACAAGACCTCTGACAGCA-3';
Caspase-1, reverse: 5'-TTGGGCAGTTCTTGGTATTC-3'.

The amplification conditions were as follows: 95˚C for 5 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min, and 72°C for 30 s followed by a final extension step at 72°C for 10 min. GAPDH was used as the control. The relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method [17]. The experiments were performed in triplicate.

Statistical analysis

Data were expressed as the mean±standard deviation (SD) of at least three independent experiments. Statistical analysis of comparisons between groups was performed with Student's t-test or one-way analysis of variance (ANOVA) using SPSS version 18.0 software (SPSS Inc, Chicago, IL, USA). A p-value of <0.05 was considered to indicate statistical significance.

Results

The effects of propofol in the rat model of pain due to inflammation following subcutaneous injection with complete Freund's adjuvant (CFA)

Several behavioral tests were performed on the rats after they were treated with propofol and CFA, including the mechanical withdrawal threshold (MWT), thermal withdrawal latency (TWL), and frequency responses to cold stimulation. CFA significantly reduced the MWT, TWL, and frequency responses to cold stimulation of rats ($p<0.05$). However, propofol significantly improved the MWT, TWL, and frequency responses to cold stimulation of rats at 1 d, 4 d, 7 d, and 14 d after treatment ($p<0.05$) (Figure 1A–1C).

Propofol reduced the levels of proinflammatory factors in the serum the rat model

Four days after CFA treatment, the effect of propofol on the expression levels of proinflammatory mediators in the rat

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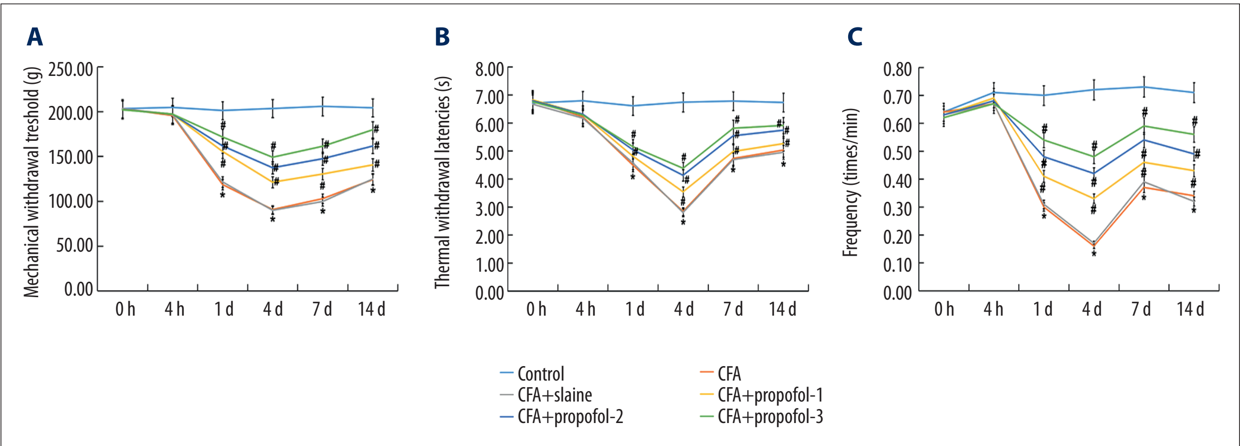

Figure 1. The effect of propofol on behavior in the rat model of pain due to inflammation following injection with complete Freund's adjuvant (CFA). (**A**) Evaluation of the mechanical withdrawal threshold (MWT) after CFA treatment in the rat model. (**B**) Detection of the thermal withdrawal latency (TWL) after CFA treatment in the rat model. (**C**) Frequency responses to cold stimulation after CFA treatment in the rat model. Sprague-Dawley rats were randomly assigned into six groups (n=10 per group). The Control group: rats were injected with 100 µl normal saline in the tail vein once every day for four consecutive days; the CFA group: 100 µl CFA was injected into the paw of Sprague-Dawley rats; the CFA+saline group: the rats were injected with the same volume of normal saline with propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA; the CFA+propofol-1 group: the rats were injected with 10 mg/kg propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA; the CFA+propofol-2 group: rats were injected with 20 mg/kg propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA; the CFA+propofol-3 group: the rats were injected with 40 mg/kg propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA. * p<0.05 *vs.* the Control group; # p<0.05 *vs.* the CFA group.

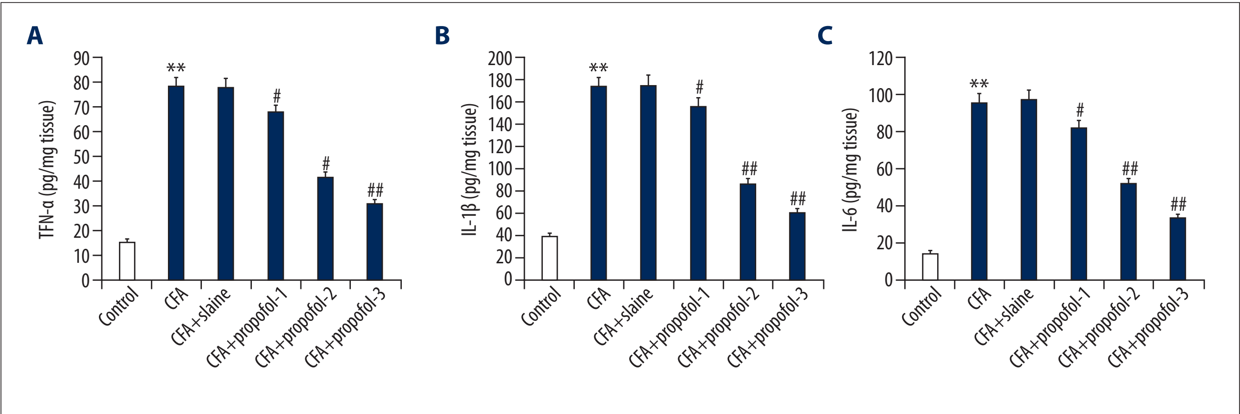

Figure 2. The effect of propofol on serum levels of proinflammatory factors in a rat model of pain due to inflammation following injection with complete Freund's adjuvant (CFA). (**A**) An enzyme-linked immunosorbent assay (ELISA) detected the expression of TNF-α in rat serum on the 4th day after CFA injection. (**B**) ELISA detected the expression of IL-1β in rat serum on the 4th day after CFA injection. (**C**) ELISA detected the expression of IL-6 in rat serum on the 4th day after CFA injection. Sprague-Dawley rats were randomly assigned into six groups (n=10 per group). The Control group: rats were injected with 100 µl normal saline in the tail vein once every day for four consecutive days; the CFA group: 100 µl CFA was injected into the paw of Sprague-Dawley rats; the CFA+saline group: the rats were injected with the same volume of normal saline with propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA; the CFA+propofol-1 group: the rats were injected with 10 mg/kg propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA; the CFA+propofol-2 group: rats were injected with 20 mg/kg propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA; the CFA + propofol-3 group: the rats were injected with 40 mg/kg propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA. * p<0.05 *vs.* the Control group; # p<0.05 *vs.* the CFA group. ** p<0.01 *vs.* the Control group; #, ## p<0.05, 0.01 *vs.* the CFA group.

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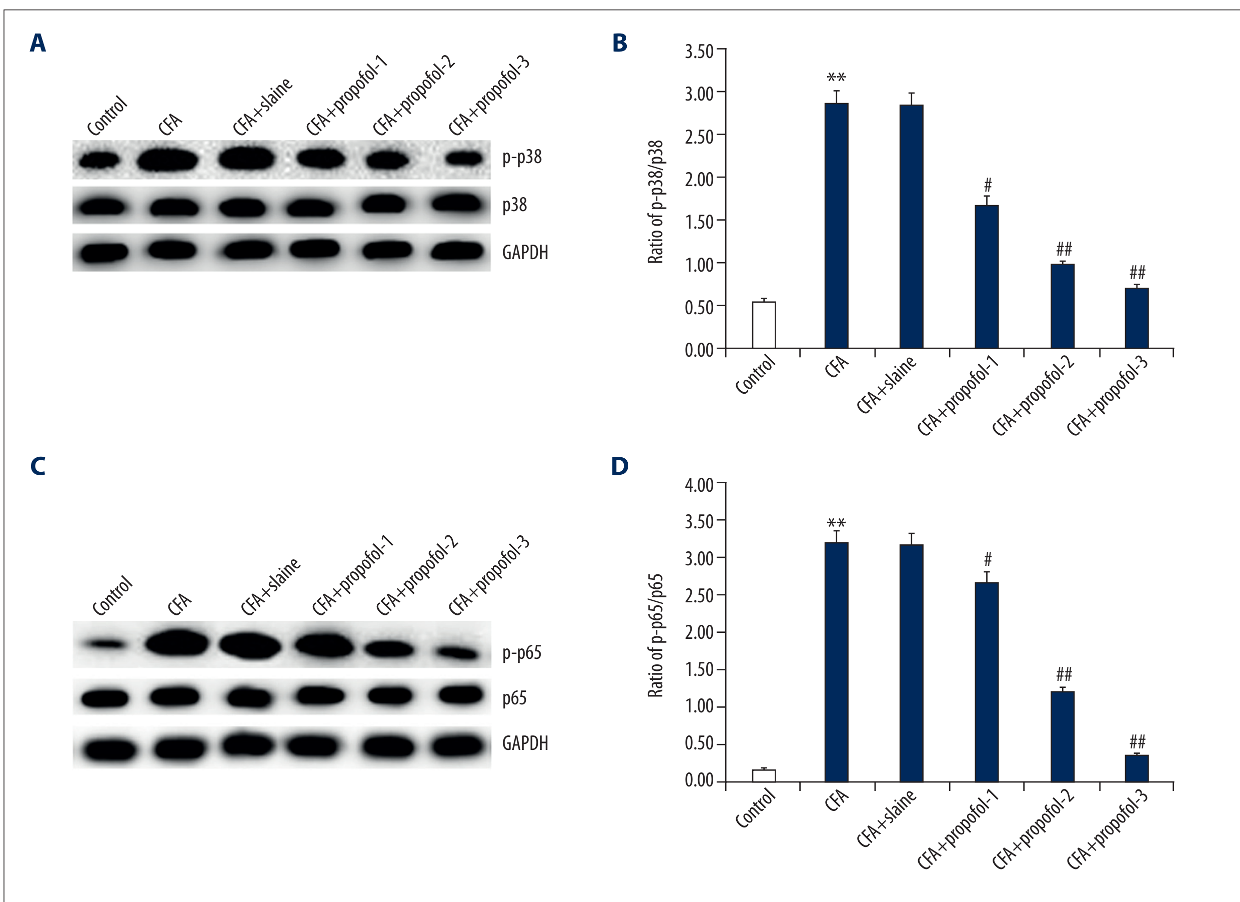

Figure 3. The effect of propofol on p38MAPK-NF-κB pathway signaling in a rat model of pain due to inflammation following injection with complete Freund's adjuvant (CFA). (**A**) The relative expression of p-p38 and p38protein in rat spinal cord tissues were measured by Western blot. (**B**) The ratio of p-p38/p38 was calculated and presented. (**C**) The protein expression of p-p65 protein in rat spinal cord tissues were measured by Western blot. (**D**) The ratio of p-p65/p65 was calculated and presented. Sprague-Dawley rats were randomly assigned into six groups (n=10 per group). The Control group: rats were injected with 100 µl normal saline in the tail vein once every day for four consecutive days; the CFA group: 100 µl CFA was injected into the paw of Sprague-Dawley rats; the CFA+saline group: the rats were injected with the same volume of normal saline with propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA; the CFA+propofol-1 group: the rats were injected with 10 mg/kg propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA; the CFA+propofol-2 group: rats were injected with 20 mg/kg propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA; the CFA+propofol-3 group: the rats were injected with 40 mg/kg propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA. * p<0.05 *vs.* the Control group; # p<0.05 *vs.* the CFA group. ** p<0.01 *vs.* the Control group; $^{\#}$, $^{\#\#}$ p<0.05, 0.01 *vs.* the CFA group.

model were evaluated by enzyme-linked immunosorbent assay (ELISA). CFA injection significantly reduced the serum levels of proinflammatory cytokines, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 compared with the Control group (p<0.05) (Figure 2A–2C).

Propofol reduced inflammatory pain following CFA injection by reducing p38MAPK-NF-κB pathway activation

To investigate the signaling pathways in the rat model after CFA injection, p-p38, p38, p65, and p-p65 levels in rat spinal cord tissues were detected by Western blot. The results showed that protein expression levels of p-p38 (Figure 3A), p-p65 (Figure 3C), and the ratio of p-p38/p38 (p<0.01) (Figure 3B) and p-p65/p65 (p<0.01) (Figure 3D) in the Control group were significantly lower than that in the CFA group. However, propofol significantly reduced p-p38 and p-p65 levels in spinal cord tissues in the CFA rat model. Propofol significantly inhibited the ratio of p-p38/p38 (p<0.05) (Figure 3B) and p-p65/p65 (p<0.05) (Figure 3D).

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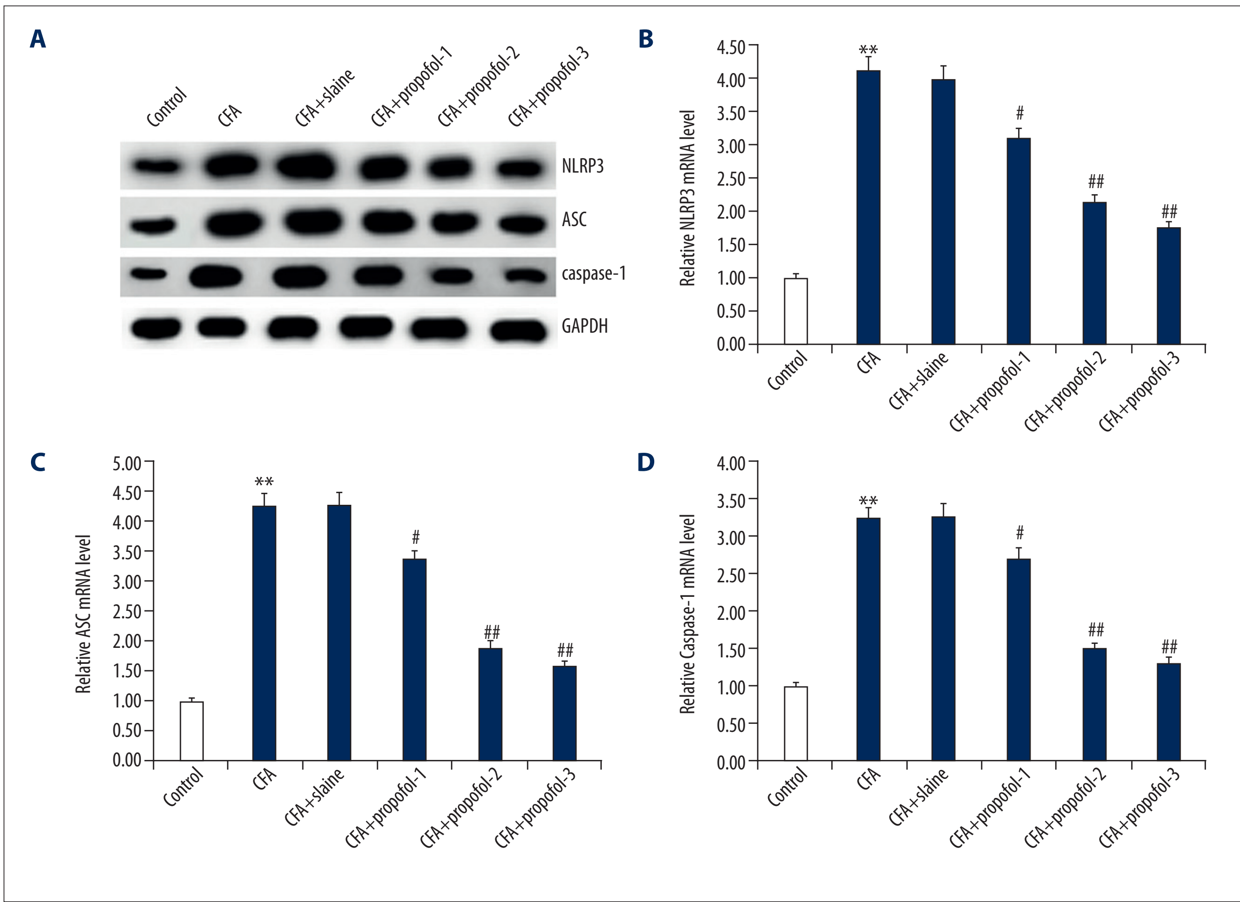

Figure 4. The effect of propofol on the NOD-like receptor family protein 3 (NLRP3) inflammasome in a rat model of pain due to inflammation following injection with complete Freund's adjuvant (CFA). (**A**) Western blot was performed to detect the levels of NLRP3, apoptosis-associated speck-like protein (ASC), and caspase-1 in rat spinal cord tissues. (**B–D**) The mRNA levels of NLRP3, ASC, and caspase-1 in inflammatory pain model rats were determined by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Sprague-Dawley rats were randomly assigned into six groups (n=10 per group). The Control group: rats were injected with 100 µl normal saline in the tail vein once every day for four consecutive days; the CFA group: 100 µl CFA was injected into the paw of Sprague-Dawley rats; the CFA+saline group: the rats were injected with the same volume of normal saline with propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA; the CFA+propofol-1 group: the rats were injected with 10 mg/kg propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA; the CFA+propofol-2 group: rats were injected with 20 mg/kg propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA; the CFA+propofol-3 group: the rats were injected with 40 mg/kg propofol through the tail vein, and 1 hour later, the rats were treated with 100 µl CFA. * p<0.05 *vs.* the Control group; # p<0.05 *vs.* the CFA group. ** p<0.01 *vs.* the Control group; #, ## p<0.05, 0.01 *vs.* the CFA group.

The effects of propofol on the NOD-like receptor family protein 3 (NLRP3) inflammasome in inflammatory pain model rats

To investigate the mechanism for the effects of propofol on CFA-induced pain associated with inflammation, NLRP3, apoptosis-associated speck-like protein (ASC), and caspase-1 mRNA and protein expression in rat spinal cord tissues were measured. CFA significantly increased the protein expression of NLRP3, ASC, and caspase-1 in rat spinal cord tissues compared with the Control group. However, propofol treatment reduced the protein expression of NLRP3, ASC, and caspase-1 in the spinal cord tissues in the rat model (Figure 4A). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis showed that the upregulation of NLRP3, ASC, and caspase-1 mRNA expression in the spinal cord tissues of CFA induced rats were significantly reduced by propofol treatment (p<0.05) (Figure 4B–4D).

Discussion

Propofol is commonly used as an intravenous anesthetic [18]. Recently, there have been several reports on its effects in

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diseases including Parkinson's disease, epilepsy, asthma, and ischemic heart disease, but there have been few reported studies on the effects of propofol on pain associated with inflammation [19–22]. Propofol has been shown to reduce apoptosis and inflammation induced by sevoflurane in human neuroglioma cells through the NF-κB signaling pathway [23]. This study aimed to investigate the role of propofol in a rat model of pain associated with inflammation following injection with complete Freund's adjuvant (CFA) into the plantar surface of the right back paw of Sprague-Dawley rats. The findings of the present study showed that propofol reduced CFA-induced inflammatory pain in rats from the results of behavioral testing, including the mechanical withdrawal threshold (MWT), thermal withdrawal latencies (TWL), and frequency responses to cold stimulation.

The findings from a previous study showed that overexpression of proinflammatory factors promoted apoptosis and further aggravated pain associated with inflammation [24]. In the present study, serum levels of proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA). The results showed that propofol significantly inhibited the serum levels of these proinflammatory factors in the rat model. The findings from the present study were consistent with those from a previous study, in which inflammation-associated factors in rats were significantly increased after injection of CFA [25].

The present study investigated the activity of p38 MAPK and NF-κB, which are two key molecules of intracellular inflammatory signaling pathways. The signaling molecule p38 MAPK is central in inflammatory activation mechanisms and is activated by phosphorylation in the cell nucleus to induce the transcription and expression of proinflammatory factors, which may be result in pain associated with inflammation. Also, in inflammation, the NF-κB signaling pathway is activated [26]. In the present study, levels of p-p38 and p-p65 in the p38MAPK-NF-κB signaling pathway were detected by Western blot, which showed that p-p38 and p-p65 expression increased following injection of CFA, and were reduced after treatment with propofol.

The NOD-like receptor family protein 3 (NLRP3) inflammasome is associated with inflammatory pain [27,28]. In the present study, the mRNA levels of genes and the proteins associated with the NLRP3 inflammasome were detected by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and Western blot, respectively. The levels of NLRP3, apoptosis-associated speck-like protein (ASC), and caspase-1 were significantly increased in the rat model that underwent subcutaneous CFA injection. These effects were reversed in the propofol treatment group. These findings indicated that propofol effectively relieved pain associated with inflammation and modulated the levels of the genes associated with the p38MAPK-NF-κB pathway and NLRP3 inflammasome.

This preliminary study had some limitations. In this rat model, the effects of only three doses of propofol were evaluated, 10 mg/kg, 20 mg/kg, and 40 mg/kg. The effect of further doses of propofol on inflammatory pain induced by CFA injection should be studied further. In the rat model, propofol reduced CFA-induced inflammatory pain and inhibited the inflammatory response by regulating the p38MAPK-NF-κB pathway and the NLRP3 inflammasome. These preliminary findings in an animal model require further *in vivo* studies and mechanistic studies before conclusions can be made regarding the potential role of propofol in the clinical management of pain associated with inflammation.

Conclusions

This study aimed to investigate the molecular mechanisms associated with the effects of propofol in a rat model of pain due to inflammation following subcutaneous injection with complete Freund's adjuvant (CFA). In this animal model, propofol reduced CFA-induced pain and inhibited the inflammatory response through the p38MAPK-nuclear factor-κB (NF-κB) pathway and the NOD-like receptor family protein 3 (NLRP3) inflammasome.

Conflict of interest

None.

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