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Propofol Relieves Neuropathic Pain Caused by Chronic Contraction Injury in the Spinal Cord

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ABSTRACT

The objective of this study is to explore the regulating pathway of propofol in alleviating neuropathic pain caused by chronic contraction injury. Forty-five rats were randomly divided into three groups: blank group (untreated, 3 groups, n=15), isoflurane group (general anesthesia of plantar incision, inhalation of 2.5% isoflurane, 3 groups, n=15), propofol group (general anesthesia of plantar incision, intravenous infusion of propofol to the lateral tail vein through the implanted catheter with the infusion rate of 1.5 mg kg^{-1} min^{-1}, 3 groups, n = 15). The paw withdrawal threshold was used to evaluate the mechanical pain before and after the incision. L3-L5 was taken 1h after incision. The phosphorylation level of GluN2b, p38MAPK, ERK, JNK and EPAC was measured by Western blot and immunofluorescence. We found that the mechanical pain induced by plantar incision peaked at 1h after surgery and lasted for 3 days. Compared with the isoflurane group, the mechanical pain in propofol group was significantly reduced within 2h after incision (P<0.05). In the propofol group, the phosphorylation level of GluN2B, p38MAPK and EPAC1 was significantly decreased (P<0.05). The number of dorsal spinal cord neurons co-expressed with EPAC1 and c-fos was significantly decreased in the propofol group after surgery (P<0.05). To conclude, propofol could reduce postoperative pain response in animals and inhibit the glun2b-p38mapk / EPAC1 signaling pathway in the spinal cord.

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Authors' Contribution
YC and JZ collected the samples. YC and FL analysed the data. JZ and FL conducted the experiments and analysed the results. All authors discussed the results and wrote the manuscript.

Key words
Propofol, GluN2B-p38MAPKEPAC1, Chronic contraction injury, Neuropathic pain

INTRODUCTION

The treatment of postoperative pain is a challenge in clinical practices. More than half of patients suffer from insufficient pain relief and 10%-50% of them suffer from persistent pain after surgery. Propofol is a commonly used general anesthetic for induction and maintenance of general anesthesia (Cattano *et al*., 2008; Chen *et al*., 2017). Compared with inhalation anesthesia, total intravenous anesthesia (TIVA) can reduce postoperative acute pain. The use of propofol as an auxiliary analgesic is still controversial, and its potential mechanism in postoperative analgesia is mostly unknown (Deng *et al*., 2016). After surgery, patients will have acute postoperative pain characterized by mechanical allergy (walking, coughing or touching the surgical wound aggravates the pain). N-methyl-D-aspartic acid (NMDA) receptor is widely expressed in the central nervous system, playing an important role in the generation and maintenance of central sensitization and causing hyperalgesia and ectopic pain (Fan and Leng, 2020).

NMDA receptors include GluN1 and GluN2 subunits, and the latter includes four types of subunits: GluN2A, 2B, 2C and 2D. GluN2B subunit is mainly expressed in lamina I synapse of spinal dorsal horn, and plays a key role in nociceptive signal transmission (Ferron *et al*., 2016). It has been reported that P-P 38 MAPK phosphorylation induced by plantar incision can increase the expression of exchange protein directly activated by cAMP binding protein (EPAC) in dorsal root ganglion (DRGs), thus promoting the occurrence of nociceptive hypersensitivity (Ferron *et al*., 2016). EPAC is a newly discovered cAMP target protein, which plays a key role in the development of inflammation and postoperative hyperalgesia as an essential effector protein (Gaamouch *et al*., 2016). Plantar injection of EPAC agonist can cause long-term mechanical hyperalgesia. Intraoperative inhibition of p-p38MAPK not only prevents the expression of EPAC in neurons and the sensitization of pain receptors induced by EPAC, but also blocks the development of pain sensitivity after subacute surgery (Gutierrez-Vargas *et al*., 2014). The increase of EPAC expression in neurons caused by activation of

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p38MAPK is the key factor leading to plantar incision or long-term nociceptive hypersensitivity after inflammation (Han *et al*., 2020). The upstream molecules that activate p38MAPK/EPAC cascade in postoperative hyperalgesia are still unknown (Hong *et al*., 2018; Kim *et al*., 2018). In this study, we explored the regulating effect of propofol on p38MAPK/EPAC pathway in L3-L5 neurons after surgical incision, so as to further understand the analgesia mechanism of propofol on postoperative pain.

MATERIALS AND METHODS

Experimental methods

Adult male SDrats, weighing about 250-300g, were kept in cages according to the standard 12h light/dark cycle, and fed and drank freely. Rats were randomly divided into three groups, namely blank group (untreated, 3 groups, n=15), isoflurane group (general anesthesia of plantar division, inhalation of 2.5% isoflurane, group 3, n=15), propofol group (receiving general anesthesia of plantar infusion, intravenous infusion of propofol to lateral tail vein through implanted catheter with infusion rate of 1.5 mg kg^{-1} min^{-1}, group 3, n=15). According to the guidelines for dose conversion between animals and humans, the dose of propofol for animals is equivalent to the human dose of 0.2 mg kg^{-1} min^{-1} needed to maintain general anesthesia. Isoflurane group (2.5%) or propofol group (1.5 mg kg^{-1} min^{-1}) was given general anesthesia for 30 min. Half an hour recovery time was allowed after plantar incision, and all rats met the recovery standard (i.e., recovery of upright reflection). The mechanical joint pain of the contralateral and ipsilateral paws was measured before and 72h after implantation incision by research assistants who were not involved in providing general anesthesia and performing plantar incision. Mechanical ectopic pain occurred 3 days after plantar incision. According to previous studies, the decrease of paw withdrawal threshold (PWT) caused by plantar incision has no difference after 3 days.

Plantar incision

Plantar incision was used as the postoperative pain model. Under general anesthesia with isoflurane or propofol, the soles of the right hind paws were disinfected with 10% povidone iodine solution and 75% ethanol. A 1cm longitudinal incision was made with No.11 blade, starting from 0.5cm away from the distal end of the talus, extending to the toe and passing through the skin and fascia of the sole of the foot. The flexor digitorum brevis was raised and cut twice. The origin and insertion of muscles remained intact. Skin suture (5-0 nylon) was performed. Plantar incision was completed within 30min minutes after surgery. After surgery, rats were placed in a constant temperature cage for 30 min, and anesthesia state and righting reflex state were restored.

Evaluation of mechanical pain

Mechanical resistance to pain was evaluated by measuring mechanical threshold (animal's response to harmless mechanical stimulation). The mechanical threshold was measured by testing the retreat reaction of the right hind paw to von Frey fibers of an electric Frey instrument (IITC Life Sciences, Lindishan, California, U.S.A). Rats were placed on the metal mesh floor where there was a transparent plastic dome. The soles of paws could be contacted through the metal mesh. Each rat adapted to the environment 30min minutes before the experiment. During the evaluation, a von Frey fiber was pressed vertically against the center of the plantar surface of the hind legs with continuous force. Withdrawing the front paws within 6~8s was a positive reaction. Any hind paw movement caused by movement was not recorded as a positive reaction. If the mouse did not retract its paw, a harder filament would be used until a positive reaction was observed. In this study, the range of probes was 0.4g-74g. The force (in grams) was displayed on the screen of the electrotherapy instrument and recorded in each positive reaction. Each rat was evaluated 3 times with an interval of about 1min. The average value of three repetitions was used as the final mechanical threshold.

Western bloting

Lumbar dorsal horn of L3-L5 segments was collected 1h after surgery. 1% protease inhibitor mixture (50mm Tris-HCl, pH7.5, 0.5% SDS, 5% 2-mercaptoethanol) was used to homogenize the tissue. The denatured supernatant was boiled for 5min. The protein was separated and transferred, and then detected by anti-phosphoprotein or pan-GluN2B, ERK1/2, p38 MAPK, JNK, Epac1/2 and endogenous protein GAPDH. After that, the membrane was incubated with the second goat anti-rabbit or mouse IgG at room temperature for 1h. After enhanced chemiluminescence incubation, X-ray films showed protein band. The gray value of the band was analyzed by ImageJ software.

Immunofluorescence staining

Rats were anesthetized with pentobarbital sodium 1h after surgery, and 4% paraformaldehyde solution was perfused through cardiovascular system. Lumbar segments of L3-L5 were collected, dehydrated and buried in -80°C tissue freezing solution. Cryosections was transversely placed in 15μm cryostat. Then, 10% normal goat serum was used to block slices of phosphate (PBS) at room temperature for 1h, followed by hatching major antibodies (S) including mouse anti-c-Fos (1:100, Abcam, Cambridge, UK), mouse anti -Epac1-mixed, rabbit anti -NeuN antibody (1:500), rabbit anti -Epac2 antibody

(1:100) and mouse anti -NeuN mixed antibody (1:500) and incubating overnight at 4℃. After PBS washing, the slices were incubated with fluorescently labeled secondary antibody (1:500, goat anti-mouse IgG labeled Alexa Fluor 488 or 568, goat anti-rabbit IgG labeled Alexa Fluor 568 or 488, Thermofisher Scientific Corporation). The slices were installed with the fixed medium of 4', 6-diamino-2-phenylindole and dihydrochloride (DAPI) (Burlingame Vector Laboratory, California, U.S.A). Immunoreactive cells were identified under confocal microscope. ImageJ software (Version 1.52c, NIH, U.S.A) was used to count double-labeled immunohistochemical cells of Epac1 or Epac2 using NeuN or c-Fos using DPAI. The cells were expressed as the percentage in total cells under the same ROI.

Statistical analysis

All data in this study are expressed by means±standard error (SEM). ImageJ software was normalized to the corresponding loading control bands, and Western blot protein band density was measured. The calculation was performed by GraphPad Prism software (GraphPad software Inc, CA, USA). Two-way ANOVA and Tukey's multiple comparison (multiple comparison table) were used to analyze the time process data of behavior test; one-way ANOVA was adopted in other analysis. In all statistical comparisons, *p* value less than or equal to 0.05 was considered statistically significant.

RESULTS

Propofol could inhibit mechanical pain caused by plantar incision

The ipsilateral hind paw narrowing threshold (PWT) in isoflurane group was lower than that at baseline (55.2±0.8g), and the narrowing threshold was the lowest 1h after plantar incision. PWT gradually recovered to baseline level from 2h after surgery, but there was a significant difference between 72h after incision and baseline (P<0.05). The PWT reached the lowest 1h after incision. However, the ipsilateral PWT completely recovered after 72h. The PWT value of ipsilateral side was lower than that of contralateral side in isoflurane group and propofol group (Table I).

We collected cell signals from the L3-L5 plantar incision from spinal cord tissues of each treatment group 1h after treatment, and assessed the difference in pain induced by mechanical contact between the two groups was the greatest after this time point.

Propofol could inhibit the expression of phosphorylated GluN2B in spinal cord induced by plantar incision

Compared with simple group, isoflurane group showed a higher level of phosphorylated GluN2B (p-GluN2B) protein in ipsilateral dorsal horn 1h after plantar incision (P<0.05). Compared with blank group, propofol group had a higher expression of p-GluN2B in the ipsilateral dorsal horn (P<0.05). The expression of p-glun2b in propofol group was lower than that in isoflurane group. There is no statistical difference in the contralateral lumbar dorsal angle among all groups (P>0.05). The results were shown in Table 1I.

Propofol inhibited the activation of p38 MAPKs in spinal cord after plantar incision

One hour after incision, the ratios of phosphorylated p38MAPK (p-p38) and pan-p38 MAPK in ipsilateral dorsal horn of L3-L5 lumbar spinal cord in isoflurane group was higher than that in blank group (P<0.05). The expression ratios of p-p38 and pan-p38 MAPK in ipsilateral dorsal horn of isoflurane group were lower than those of isoflurane group ($P<0.05$). The results were shown in Table 1. Compared with blank group (0.96±0.03), the ratios of p-ERK and pan-ERK in isoflurane group (1.32±0.04) and propofol Group (1.28 0.05) were higher, but there was no significant difference in p-ERK ratio between isoflurane group and isoflurane group ($P>0.05$, Table 1). The ratios of p-JNK to pan-JNK in ipsilateral spinal cord had no difference among the three groups ($P>0.05$). The expression of p-p38, p-ERK and p-JNK in contralateral dorsal horn had no difference among the three groups ($P>0.05$, Table 1).

Table I. Effects of propofol and isoflurane on paw withdrawal threshold after plantar incision.

Time (h)	Baseline	1h	2h	5h	24h	48h	72h	F	
Ipsilateral incision									
propofol	55.2±0.8	33.3±1.1	43.3±1.3	40.2±2.6	41.2±6.1	40.1±2.6	50.4±0.6	25.369	0.012
Isoflurane	55.1±0.6	20.8±4.3	30.1±2.5	54.2±3.1	55.3±4.3	55.4±4.6	47.4±0.8	39.812	0.002
Contralateral incision									
Propofol	55.1±0.5	42.1±2.3	55.1±2.2	35.6±4.1	38.3±4.2	37.2±5.4	35.4±5.2	40.251	0.001
Isoflurane	55.2±0.8	55.1±4.2	42.3±2.6	54.4±2.9	55.3±2.5	55.6±5.6	55.1±4.6	19.537	0.035
F	1.256	80.765	76.541	79.042	100.861	70.952	105.871	-	-
P	0.452	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	-	-

Table II. Expression of p-GluN2B, pan-GluN2B, p38 MAPK, pan-p38 MAPK, p-ERK, pan-ERK and Epac 1 in ipsilateral and contralateral L3-L5 after planar incision (n=15).

Subject	Incision	Blank group	Isoflurane group	Propofol group	F	p
p-GluN2B/ pan-GluN2B	Ipsilateral	0.39±0.09	1.14±0.07	0.67±0.01	102.345	0.001
	Contralateral	0.52±0.03	0.49±0.04	0.53±0.06	5.452	0.235
p-38B/ pan-38	Ipsilateral	0.70±0.06	1.16±0.02	0.88±0.10	28.045	0.012
	Contralateral	1.03±0.04	0.98±0.03	1.09±0.02	5.047	0.101
p-ERK/pan-ERK	Ipsilateral	0.96±0.03	1.32±0.04	1.28±0.05	103.092	0.005
	Contralateral	1.05±0.04	1.11±0.02	0.99±0.02	4.054	0.112
Epac1	Ipsilateral	1.20±0.04	1.52±0.04	0.65±0.05	30.854	0.002
Epac2	Contralateral	0.821±0.08	1.28±0.01	0.801±0.08	5.306	0.095

Propofol inhibited the expression of EPAC1 after plantar incision, but did not inhibit the expression of EPAC2

As shown in Table II and Figure 1, compared with blank group, EPAC1 in isoflurane group was increased (*P<0.05*). Compared with isoflurane group, EPAC1 was decreased in propofol group (*P<0.05*). There was no significant difference in the expression of EPAC1 in contralateral dorsal horn among different groups (n=15, *P>0.05,* 0.821±0.08, 1.28±0.01 and 0.801±0.08 for blank group, isoflurane group and propofol group respectively). The expression of EPAC2 in dorsal spinal cord of each group did not change after plantar incision (*P>0.05*).

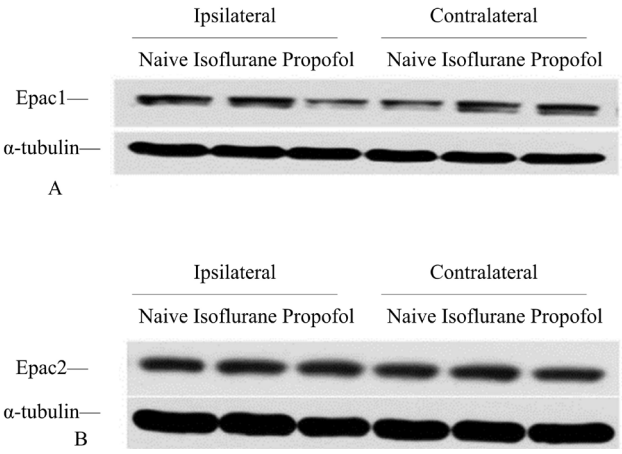

Fig. 1. Expression of EPAC1 (A) and EPAC2 (B).

Propofol prevented c-Fos expression in spinal cord induced by plantar incision

Compared with blank group, the c-Fos positive cell number and IL3-L5 segment (L3-L5) of DAPI double label plate in in isoflurane group were increased after 1h of plantar Incision. Compared with isoflurane group, the number of c-Fos positive cells labeled with DAPI in propofol group was decreased (*P<0.05*). Compared with blank group, the number of c-Fos positive cells in slice III-V was increased in isoflurane group and propofol group 1h after incision (*P<0.05*). There was no significant difference in the number of c-Fos positive cells between propofol group and isoflurane group (*P>0.05*). This indicated that propofol selectively inhibited nociceptive signal transmission in lumbar dorsal horn after plantar incision. As shown in Table III and Figure 2.

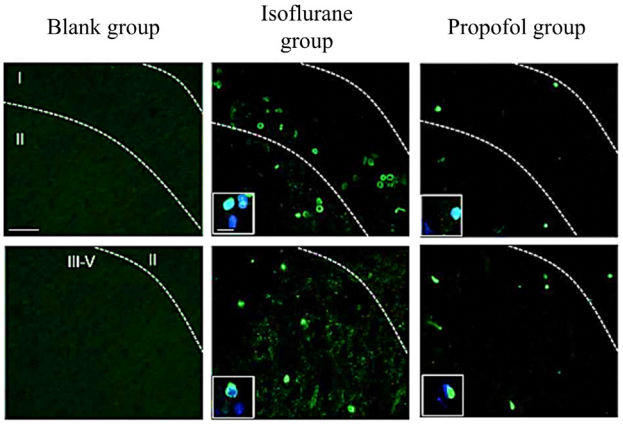

Fig. 2. Distribution of c-Fos positive cells in L3-L5 of blank group, isoflurane group and propofol group.

Table III. Distribution results of c-Fos positive cells in L3-L5 of blank group, isoflurane group and propofol group (n=15).

Group	Lamina I	Laminae III-V
Blank group	2.30±0.60	2.10
Isoflurane group	13.20±0.50	4.86±0.03
Propofol group	4.70±0.20	5.66±0.03
F	59.056	30.382
P	<0.001	0.001

DISCUSSION

In this study, by comparing the effects of propofol infusion and inhalation of isoflurane on NMDAreceptor of GluN2B subunit content and MAPK/EPAC pathway downstream in postoperative pain model of rats after plantar incision, it is speculated that propofol played an anti-injury role by regulating GluN2B /MAPK/EPAC cascade at L3-L5 level after surgery.

In this study, the density ratio of ipsilateral phosphorylated GluN2B to panGluN2B increased within 1h after incision. Propofol inhibited GluN2B phosphorylation 1h after surgery on the ipsilateral L3-L5 dorsal horn, but the contralateral side had no change. It has been reported that GluN2B is one of the targets of propofol inhibiting NMDAreceptor activation and regulating Ca2+ influx through slow calcium channel in cultured hippocampal neurons (Liu *et al*., 2018; Pang *et al*., 2010). Propofol inhibits rats status epilepticus induced by lithium-pilocarpine *in vivo* by down regulating GluN2Bsubunits expression (Pernow *et al*., 2015; Shu *et al*., 2015). However, the information about the effect of propofol on spinal cord neurons is rather limited (Üner *et al*., 2015). The results show that intraperitoneal injection of propofol has anti-injury effect in rate writhing with acetic acid, while intrathecal injection of NMDA agonist can reverse this effect and NMDAreceptor antagonist can enhance this effect. This study not only confirms the above findings, but also proves that NMDAreceptor GluN2B subunits in the spinal cord is an important target for reducing mechanical pain by intravenous injection of propofol compared with inhalation of isoflurane. However, it is still unclear whether propofol can inhibit GluN2B by directly binding to NMDAreceptor or indirectly bypassing it (Wong *et al*., 2019).

In neurons, the influx of calcium ions through NMDAreceptor leads to the activation of MAPK pathway. Phosphorylation of MAPKs in the spinal cord, such as p38 and ERK1/2, is involved in the occurrence and maintenance of mechanical hypersensitivity in acute postoperative pain model (Zhong *et al*., 2020). Studies have shown that the phosphorylation of p38 MAPK in dorsal spinal neurons and microglia is increased 1-3 days after plantar incision (Zhou *et al*., 2020; Zhu *et al*., 2019). Intrathecal injection of p38 inhibitor 30min before plantar incision can alleviate the mechanical pain caused by incision. Similarly, L3-L5ERK is activated several minutes after plantar incision, while pretreatment with MEK inhibitor U0126 reduces pain response. Consistent with previous reports, we found that the activation of p38 MAPK and ERK in the spinal cord was increased 1h after plantar incision. Compared with isoflurane group, the expression of p38MAPK, rather than that of ERK1/2, activated after rats plantar incision was significantly decreased. Unlike ERK1/2, p38 MAPK is mainly activated by inflammatory cytokines. Early studies have shown that tissue injury without nerve injury after surgery can initiate a series of inflammatory cytokines, which are positively correlated with postoperative pain intensity (Cattano *et al*., 2008). The current research results showed that the analgesic effect of propofol in postoperative pain model was related to its anti-inflammatory effect. In addition, the study also showed that enhanced phosphorylation of p38MAPK played an important role in the occurrence of persistent pain of rats after spinal cord surgery.

This study showed that intravenous injection of propofol by plantar incision could reduce mechanical pain compared with inhalation of isoflurane in rats postoperative pain model. The analgesic effect of propofol may be achieved by inhibiting NMDAreceptor containing GluN2B in neurons and p38MAPK/EPAC1 signaling pathway at L3-L5 level downstream. Selective inhibition of propofol on p38 MAPK/EPAC1 and c-Fos expression on L3-L5 surface provided further evidence-based molecular and cellular mechanism for propofol as an auxiliary analgesic drug for general anesthesia.

To sum up, propofol could reduce postoperative pain response of animals and inhibit GluN2B-p38MAPK/EPAC1 signaling pathway in the spinal cord of animals.

Statement of conflict of interests

The authors have declared no conflict of interest.

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