

## Original Article

# Anesthetic isoflurane reduces neuropathic pain by inhibiting IL-1 $\beta$ -induced COX-2 activation and CCL2 release in rat trigeminal ganglia cells

Jun Zhang<sup>1\*</sup>, Zhiqiang Gu<sup>2\*</sup>, Lian Xue<sup>3</sup>, Tieli Dong<sup>1</sup>, Jianfeng Li<sup>1</sup>, Zhigang Zhuang<sup>1</sup>

Departments of <sup>1</sup>Pain Medicine, <sup>2</sup>Invasive Technology, The Second Affiliated Hospital of Zhengzhou University, Zhengzhou 450014, Henan, China; <sup>3</sup>Department of Pain Medicine, The First People's Hospital of Shangqiu, Shangqiu 476000, Henan, China. \*Equal contributors and co-first authors.

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**Abstract:** Inflammatory mediators play key roles in neuropathic pain of the trigeminal system. Anesthetic isoflurane (ISO) possesses anti-inflammatory activity. However, the inhibitory effects of ISO on inflammation-triggered neuropathic pain are unclear. In this study, we investigated the effects of 1.4% ISO on neuropathic pain in interleukin-1 $\beta$  (IL-1 $\beta$ )-challenged primary rat trigeminal ganglia cells (TGCs). Here, we found that ISO reduced the mRNA and protein levels of cyclooxygenase-2 (COX-2) and C-C motif ligand 2 (CCL2) in IL-1 $\beta$ -stimulated TGCs, as determined by quantitative real-time PCR and Western blot, respectively. Enzyme-linked immunosorbent assays showed that IL-1 $\beta$ -induced prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), CCL2, and calcitonin gene-related peptide (CGRP) release from TGCs was significantly inhibited by ISO treatment. Pretreatment with a selective inhibitor of COX-2 (parecoxib) notably attenuated IL-1 $\beta$ -induced PGE<sub>2</sub> and CGRP release in TGCs. Pretreatment with an inhibitor of CCL2 synthesis (bindarit) also reduced CGRP release in IL-1 $\beta$ -challenged TGCs. Collectively, ISO ameliorates neuropathic pain through reduction of CGRP release by inhibiting COX-2 activity and CCL2 production in IL-1 $\beta$ -treated TGCs.

**Keywords:** Isoflurane, IL-1 $\beta$ , COX-2, CCL2, trigeminal ganglia cells

## Introduction

Neuropathic pain such as migraine is one of the most devastating kinds of chronic pain and is caused by a lesion or disease of the somato-sensory system [1]. In migraine, trigeminal ganglia play a pivotal role in pain initiation and maintenance [2]. In the trigeminal system, inflammatory mediators and neuropeptides are key players in the pathophysiology of migraine [3]. Despite great improvements for neuropathic pain, current treatment strategies are still unsatisfied. Therefore, searching a novel therapy for neuropathic pain is critically important and urgently needed.

Pro-inflammatory cytokines and chemokines such as interleukin (IL)-1 $\beta$ , IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and C-C motif ligand 2 (CCL2) are involved in the development of neuropathic pain [4, 5]. IL-1 $\beta$  and TNF- $\alpha$  levels are elevated in the jugular vein during migraine attacks [6]. CCL2 production is highly increased in a neuro-

pathic pain model [7]. Cyclooxygenases (COXs) are also important peripheral mediators of inflammation and pain. The non-selective and selective inhibitors of COX-2 can alleviate migraine attacks [8, 9]. Calcitonin gene-related peptide (CGRP), a member of the calcitonin family of peptides, is widely distributed in the peripheral and central nervous systems. CGRP is thought to be the main neuromediator of trigeminal signaling, specifically in headache [10]. In particular, IL-1 $\beta$  stimulation leads to increased CGRP synthesis and release in rat trigeminal ganglia cells (TGCs) [11], indicating a link between inflammatory mediators and CGRP release in neuropathic pain.

Isoflurane (ISO) is a widely used inhaled anesthetic with anti-inflammatory activity [12]. Previous studies demonstrated that ISO treatment significantly ameliorates zymosan-induced inflammatory responses in murine Kupffer cells by inhibiting COX-2/prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis, and pro-inflammatory cytokine and che-

mokine release [13]. However, whether ISO ameliorates inflammation-elicited neuropathic pain in rat TGCs remains unknown.

In this study, we evaluated the effects of ISO (1.4%) on neuropathic pain in IL-1 $\beta$ -stimulated primary rat TGCs. We found that ISO treatment reduced the mRNA and protein levels of COX-2 and CCL2 in TGCs with IL-1 $\beta$  stimulation. PGE<sub>2</sub>, CCL2, and CGRP production in IL-1 $\beta$ -treated TGCs was significantly inhibited by ISO administration. In addition, IL-1 $\beta$ -induced CGRP release was hindered by a selective inhibitor of COX-2 activation (parecoxib) or CCL2 synthesis (bindarit) in TGCs. Overall, these results suggested that ISO alleviated neuropathic pain through reduction of CGRP release by inhibiting IL-1 $\beta$ -induced COX-2 activation and CCL2 synthesis in TGCs.

## Materials and methods

### Reagents

ISO was obtained from Baxter (Baxter Healthcare Corporation, Deerfield, IL, USA). Recombinant rat IL-1 $\beta$  (R&D Systems, Minneapolis, MN, USA) was dissolved in sterile phosphate-buffered saline (PBS, 0.1 M). All reagents for cell culture were purchased from Gibco BRL (Grand Island, NY, USA). Rabbit anti-rat COX-2, CCL2, and  $\beta$ -actin polyclonal antibodies were purchased from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated anti-rabbit IgG was obtained from Chemicon (Temecula, CA, USA). A selective inhibitor of COX-2 (Parecoxib) and an inhibitor of CCL2 synthesis (bindarit) were purchased from Pfizer Pharmaceuticals, Inc. (Groton, CT, USA) and Nanjing Chemlin Chemical Industry Co. (Nanjing, Jiangsu, China), respectively. Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

### Animals

Three-day-old male Wister rats were purchased from Laboratory Animal Center of Henan Province (Zhengzhou, Henan, China). All animals were kept under standard laboratory conditions and maintained at 22-24°C with a regular 12 h/12 h day/night cycle and food and water ad libitum. The animal procedures were approved by the Ethics Committee for Animal Experimentation of Zhengzhou University and

in accordance with the guideline for the Care and Use of Laboratory Animals by the National Institutes of Health (Bethesda, MD, USA). For primary cell isolation, rats were sacrificed under anesthesia with chloral hydrate, and all efforts were exerted to minimize suffering.

### TGC isolation and culture

TGC isolation and culture were performed as previously described [14], with slight modifications. In brief, trigeminal ganglia were aseptically dissected from the 3-day-old rats. The cells were incubated in dissociation medium [Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 10 mM HEPES, 44 mM glucose, streptomycin (10  $\mu$ g/mL), penicillin (10 U/mL), 2 mM glutamine, and 100 IE insulin/L] containing collagenase/dispase (100 mg/mL) at 37°C for 90 min. The cells were then rinsed twice with PBS (0.1 M) and incubated again with trypsin/EDTA for 30 min. Subsequently, the cells were rinsed twice with PBS and once with dissociation medium, dissociated using Pasteur pipette, and pelleted through centrifugation at 2,000  $\times$ g at 20°C for 3 min. After suspension in starter medium with 1% penicillin/streptomycin, 0.25% L-glutamine, 2% B27-supplement, 25 mM glutamate, 2.5 mM calcium chloride, and 100 ng/mL NGF- $\beta$ , the cells were seeded in 24-well plates and filled with 500  $\mu$ L of starter medium at a density of  $0.5 \times 10^6$  cells/cm<sup>2</sup>. The cells were fed with neurobasal medium + B27 medium every 2nd day by replacing 50% of the medium at 37°C and 5% CO<sub>2</sub> for 6 days.

### Experimental protocols

The TGCs were seeded on six-well plates, incubated overnight, and then subjected to IL-1 $\beta$  (10 ng/mL) or vehicle (0.1 M PBS) treatment for 0.5 h. Subsequently, the cells were exposed to ISO for 0.5 h at 2 L/min in a metabolic chamber (Columbus Instruments, Columbus, OH, USA). During ISO exposure, the ISO concentration was continuously verified by sampling the exhaust gas with a Datex Capnomac (SOMA Technology Inc., Cheshire, CT, USA). The cells were continuously subjected to IL-1 $\beta$  or PBS treatment for the indicated time points. The cells exposed to room air were used as control. To investigate the inhibitory effects of parecoxib or bindarit, the TGCs were pretreated with or without parecoxib (10 nM) or bindarit (300  $\mu$ M)

for 15 min, washed out, and treated with IL-1 $\beta$  or PBS for the indicated time periods.

## 3-(4,5-di-methylthi-azol-2-yl)-2,5-diPhentetrazolium bromide (MTT) assay

Cell viability was measured using MTT assay. TGCs ( $5 \times 10^3$  cells/well) were seeded on 96-well plates and treated with different concentrations of ISO (0.7%, 1.4%, and 2.1%) for 24 h. Then, 0.5 mg/mL of MTT was added to each well and incubated at 37°C for 4 h. The supernatants were removed, and 150  $\mu$ L of dimethyl sulfoxide was added to each well. The absorbance at 490 nm was determined using a microplate reader (Bio-Tek instruments Inc., Winooski, VT, USA). The experiments were run in triplicates.

## Lactate dehydrogenase assay

The ISO toxicity on TGCs was measured by using a Thermo Scientific Pierce Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit (Thermo Scientific, Rockford, IL, USA), in accordance with the manufacturer's instructions. The assays were performed after 6-day culture to evaluate the membrane integrity of the cells, and data were recorded at a wavelength of 490 and 680 nm using a microplate reader (Bio-Tek).

## Quantitative real-time polymerase chain reaction (qPCR)

After 3 h of IL-1 $\beta$  (10 ng/mL) or PBS (0.1 M) stimulation, the total RNA from the TGCs was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was generated using SuperScript Reverse Transcriptase kit (Invitrogen). qPCR was performed with iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). PCR-specific amplification was assessed using SYBR Premix Ex Taq<sup>TM</sup> (TaKaRa, Otsu, Shiga, Japan). The relative mRNA levels of COX2 and CCL2 were calculated using the  $2^{-\Delta\Delta Ct}$  method. Glyceraldehyde-3-phosphat-dehydrogenase (GAPDH) was used as endogenous control. The primers used for PCR amplification in this study were as follows: for COX-2, forward 5'-TGATCGAAGACTACGTGCAACAC-3' and reverse 5'-CAGCA ATC TGTCTGG TGAATGAC-3'; for CCL2, forward 5'-CCAGCACCAGCAC-CAG CCAA-3' and reverse 5'-TGGATGCTCCAG-

CCGGCAAC-3'; and for GAPDH, forward 5'-AGATT GGCAATGCATGC-3' and reverse 5'-CC-TTCTTGATGTCAT CATACTTGG-3'.

## Western blot analysis

At 6 h after IL-1 $\beta$  or PBS incubation, the TGCs were lysed in lysing buffer (Beyotime, Haimen, Jiangsu, China). The proteins were separated through sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies targeting COX-2, CCL2, and  $\beta$ -actin overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibody. Protein expression was detected using an enhanced chemiluminescence assay kit (Pierce, Rockford, IL, USA). Protein band intensities were quantified using Quantity One software (Bio-Rad, USA).

## Enzyme-linked immunosorbent assay (ELISA)

At the indicated time points, the PGE<sub>2</sub>, CCL2, and CGRP levels in the supernatants of the TGCs with different treatments were measured using commercially available ELISA kits from R&D Systems Inc. (Minneapolis, MN, USA), according to the manufacturers' instructions. The PGE<sub>2</sub>, CCL2, and CGRP release was determined in pg/mL. All experiments were conducted in triplicates.

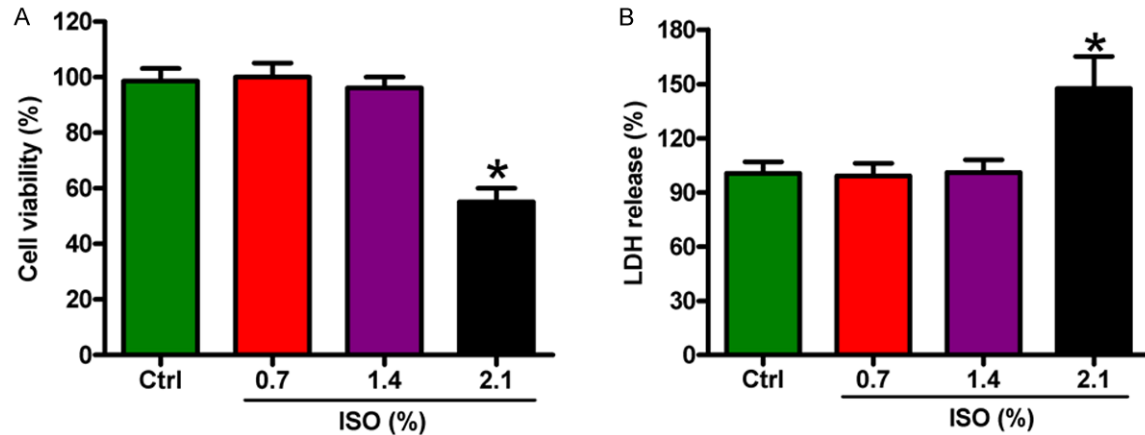
## Statistical analysis

All data were expressed as means  $\pm$  SD. Intergroup differences were determined by Student's two-tailed unpaired *t*-test or one-way ANOVA analysis, followed by Dunnett's post hoc test as appropriate. All analyses were performed by the statistical analysis software SPSS 16.0 (SPSS Inc. Chicago, IL, USA). *P* < 0.05 or *P* < 0.01 was considered statistically significant.

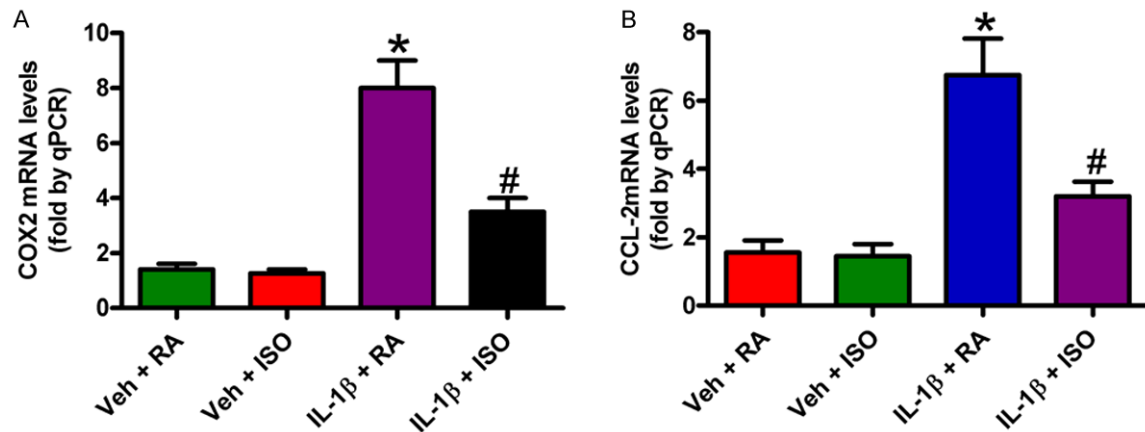
## Results

### Toxic effect of ISO on TGCs

MTT and LDH assays were performed to investigate the toxic effect of ISO on TGCs. As shown in **Figure 1A**, the two lower concentrations (0.7% and 1.4%) of ISO treatment had no significant effects on cell viability compared with the control group. However, the higher ISO concen-



**Figure 1.** Effects of ISO on the viability and LDH release of TGCs. The cells were treated with different concentrations of ISO (0, 0.7%, 1.4%, and 2.1%) for 24 h. A. Cell viability was measured using MTT assay. B. LDH release was evaluated. Data are expressed as means  $\pm$  SD. \* $P < 0.05$  versus (Vs.) Ctrl group. ISO: isoflurane; Ctrl: control.



**Figure 2.** ISO inhibited IL-1 $\beta$ -induced COX-2 and CCL2 mRNA expression in TGCs. 3 h after IL-1 $\beta$  stimulation, COX-2 and CCL2 mRNA levels of TGCs were assessed. (A and B) qPCR was conducted to evaluate COX-2 (A) and CCL2 (B) mRNA expression levels. GAPDH was used as the endogenous control. Data are expressed as means  $\pm$  SD. \* $P < 0.05$  Vs. Veh groups; # $P < 0.05$  Vs. IL-1 $\beta$  + RA group. ISO: isoflurane; Veh: vehicle; RA: room air.

tration (2.1%) significantly reduced cell viability ( $P < 0.05$ ). The toxicity of 2.1% ISO to TGCs was also confirmed by LDH assay (**Figure 1B**,  $P < 0.05$ ). These results showed that 2.1% ISO conferred toxicity to TGCs. Thus, 1.4% ISO was selected for the subsequent experiments.

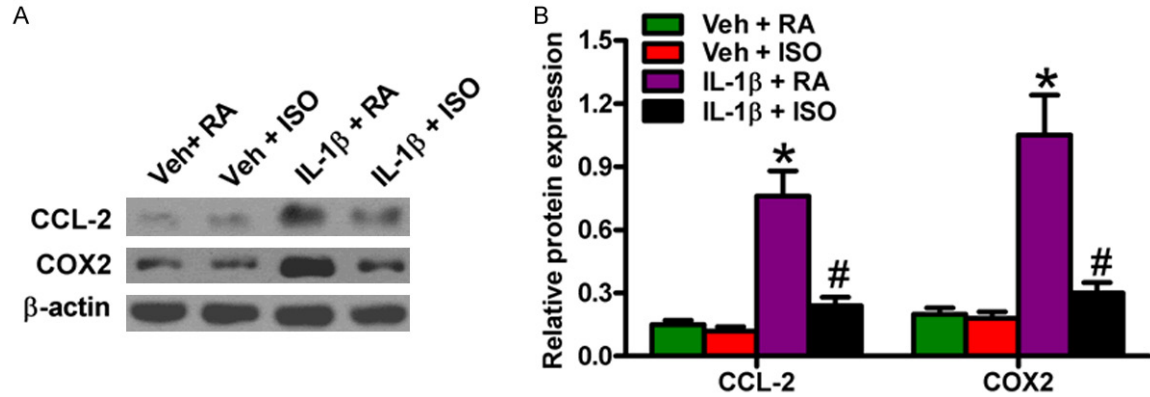
*mRNA and protein expressions of COX-2 and CCL2 were downregulated by ISO treatment in IL-1 $\beta$ -stimulated TGCs*

IL-1 $\beta$ -treated TGCs exhibited a robust increase in COX-2 mRNA level compared with the vehicle control (**Figure 2A**,  $P < 0.05$ ). Similarly, the mRNA expression of CCL2 in the TGCs was elevated by IL-1 $\beta$  stimulation (**Figure 2B**,  $P < 0.05$ ).

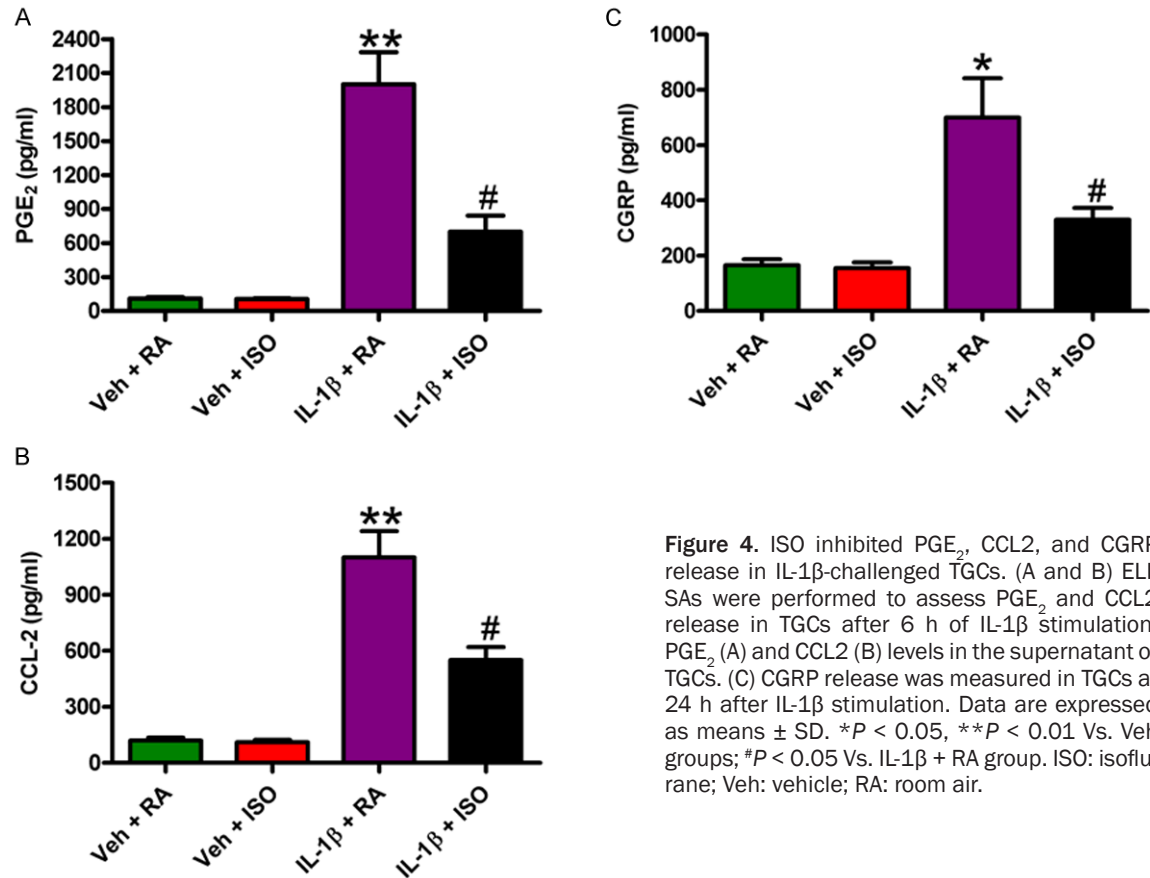
By contrast, ISO treatment remarkably reduced the increase in the mRNA levels of COX-2 and CCL2 (**Figure 2A** and **2B**,  $P < 0.05$ ). Consistently, the increased protein levels of COX-2 and CCL2 in IL-1 $\beta$ -treated TGCs were substantially reduced by ISO administration (**Figure 3A** and **3B**,  $P < 0.05$ ). These data suggested that ISO reduced IL-1 $\beta$ -increased mRNA and protein levels of COX-2 and CCL2 in TGCs.

*ISO reduced IL-1 $\beta$ -induced PGE<sub>2</sub>, CCL2, and CGPR production in TGCs*

ELISA results showed that the PGE<sub>2</sub> level in the supernatant of IL-1 $\beta$ -treated TGCs was strongly elevated compared with the vehicle control



**Figure 3.** ISO reduced IL-1 $\beta$ -enhanced COX-2 and CCL2 protein expression in TGCs. 6 h after IL-1 $\beta$  stimulation, western blot was performed to analyze COX-2 and CCL2 protein levels in TGCs. (A) Representative images of COX-2 and CCL2 protein expressions. (B) The relative protein expressions of COX-2 and CCL2 in (A) were quantified and normalized to  $\beta$ -actin intensities. Data are expressed as means  $\pm$  SD. \* $P < 0.05$  Vs. Veh groups; # $P < 0.05$  Vs. IL-1 $\beta$  + RA group. ISO: isoflurane; Veh: vehicle; RA: room air.

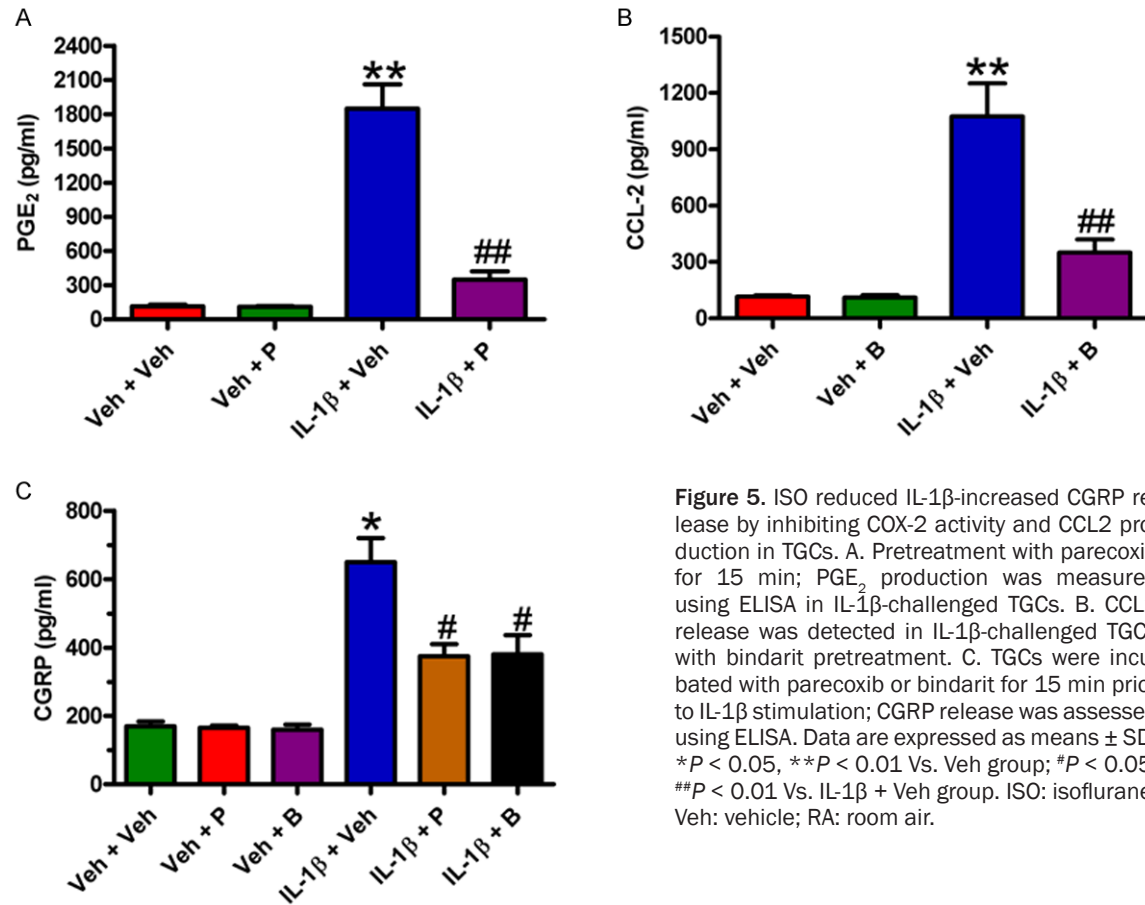


**Figure 4.** ISO inhibited PGE<sub>2</sub>, CCL2, and CGRP release in IL-1 $\beta$ -challenged TGCs. (A and B) ELISAs were performed to assess PGE<sub>2</sub> and CCL2 release in TGCs after 6 h of IL-1 $\beta$  stimulation. PGE<sub>2</sub> (A) and CCL2 (B) levels in the supernatant of TGCs. (C) CGRP release was measured in TGCs at 24 h after IL-1 $\beta$  stimulation. Data are expressed as means  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$  Vs. Veh groups; # $P < 0.05$  Vs. IL-1 $\beta$  + RA group. ISO: isoflurane; Veh: vehicle; RA: room air.

(Figure 4A,  $P < 0.01$ ). However, the increase in PGE<sub>2</sub> production was obviously reduced by ISO treatment (Figure 4A,  $P < 0.05$ ). Similarly, ISO suppressed IL-1 $\beta$ -induced CCL2 release in TGCs (Figure 4B,  $P < 0.05$ ). In addition, the

enhanced CGRP release in the IL-1 $\beta$ -treated TGCs was notably reduced by ISO treatment (Figure 4C,  $P < 0.05$ ). These results indicated that ISO reduced PGE<sub>2</sub>, CCL2, and CGRP release in the IL-1 $\beta$ -challenged TGCs.





**Figure 5.** ISO reduced IL-1 $\beta$ -increased CGRP release by inhibiting COX-2 activity and CCL2 production in TGCs. A. Pretreatment with parecoxib for 15 min; PGE<sub>2</sub> production was measured using ELISA in IL-1 $\beta$ -challenged TGCs. B. CCL2 release was detected in IL-1 $\beta$ -challenged TGCs with bindarit pretreatment. C. TGCs were incubated with parecoxib or bindarit for 15 min prior to IL-1 $\beta$  stimulation; CGRP release was assessed using ELISA. Data are expressed as means  $\pm$  SD. \* $P$  < 0.05, \*\* $P$  < 0.01 Vs. Veh group; # $P$  < 0.05, ## $P$  < 0.01 Vs. IL-1 $\beta$  + Veh group. ISO: isoflurane; Veh: vehicle; RA: room air.

#### ISO reduced CGRP release by inhibiting COX-2 activity and CCL2 synthesis in IL-1 $\beta$ -treated TGCs

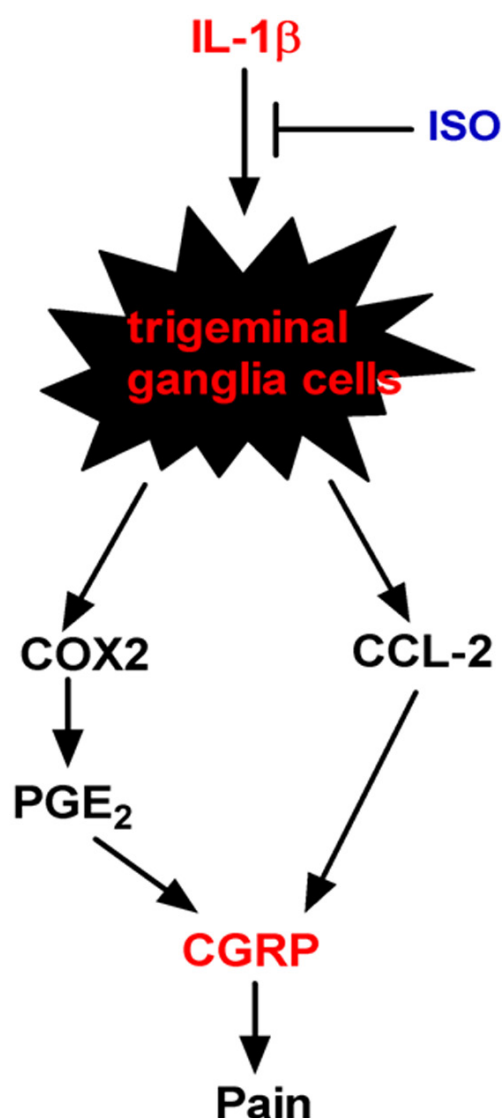
To investigate whether the inhibitory effects of ISO on CGRP release depended on the reduction of COX-2 activity and CCL2 synthesis, we pretreated TGCs with a selective COX-2 inhibitor (parecoxib) and an inhibitor of CCL2 synthesis (bindarit) prior to IL-1 $\beta$  treatment. We found that pretreatment with parecoxib or bindarit for 15 min led to notable decrease in PGE<sub>2</sub> production or CCL2 release in IL-1 $\beta$ -challenged TGCs (**Figure 5A** and **5B**,  $P$  < 0.01). CGRP release was significantly reduced in IL-1 $\beta$ -stimulated TGCs with parecoxib or bindarit pretreatment (**Figure 5C**,  $P$  < 0.05). These results indicated that ISO reduced IL-1 $\beta$ -induced CGRP release by inhibiting COX-2 activity and CCL2 synthesis in TGCs.

#### Discussion

In this study, we explored the protective effects of ISO on IL-1 $\beta$ -stimulated rat primary TGCs.

Key findings were as follows (**Figure 6**): first, the mRNA and protein levels of COX-2 and CCL2 in TGCs with IL-1 $\beta$  stimulation were reduced by ISO treatment. Second, ISO inhibited PGE<sub>2</sub>, CCL2, and CGRP production in IL-1 $\beta$ -treated TGCs. Third, IL-1 $\beta$ -induced CGRP release was reduced by parecoxib or bindarit pretreatment in TGCs. Finally, ISO reduced IL-1 $\beta$ -induced CGRP-mediated neuropathic pain by inhibiting COX-2 activation and CCL2 release in rat TGCs.

Trigeminal ganglion plays a key role in the pathophysiology of migraine and other primary headaches. TGCs have been commonly used to investigate molecular mechanisms underlying migraine pathophysiology [15]. Pro-inflammatory cytokines and chemokines have been well associated with inflammatory pain [16]. IL-1 $\beta$ -treated TGC is an established cell model of inflammatory pain [17]. IL-1 $\beta$  can activate nociceptors to induce pain hypersensitivity in a rat skin nerve preparation [18]. In inflammatory pain, IL-1 $\beta$  is known as a key mediator that induces COX-2 expression in spinal cord [17] and dorsal root ganglia cells [19]. Previous



**Figure 6.** Diagram depicting the alleviating effects of ISO on neuropathic pain in IL-1 $\beta$ -challenged TGCs.

studies showed that inflammation leads to COX-2 induction [20] and subsequent PGE<sub>2</sub> release, which sensitize peripheral nociceptor terminals and produce localized pain hypersensitivity [21]. In the present study, we demonstrated that ISO treatment significantly reduced COX-2 expression and PGE<sub>2</sub> production in IL-1 $\beta$ -challenged TGCs.

CCL2, a member of the chemokines, can specifically recruit monocytes to sites of inflammation, infection, trauma, toxin exposure, and ischemia. The biological effects of CCL2 are mediated through interaction with its receptor C-C motif receptor 2 (CCR2) [22]. It was report-

ed that mice overexpressing CCL2 in astrocytes exhibited enhanced pain sensitivity [23], whereas mice lacking CCR2 display a substantial reduction in mechanical allodynia after partial ligation of the sciatic nerve [24]. In particular, CCL2-CCR2 signaling is involved in trigeminal neuropathic pain [25]. The present study showed that CCL2 production and release were markedly increased in IL-1 $\beta$ -challenged TGCs, which were hindered by ISO treatment.

CGRP is a classical neuropeptide in the pathophysiology of migraine and other primary headaches. In the nervous system, CGRP is mainly produced by medium- and small-diameter primary afferent neurons and is important in modulating pain perception. In clinical studies, CGRP levels are increased in the plasma during migraine and cluster headache attacks [26, 27]. Previous studies showed that intravenous injection of CGRP induces a typical headache in migraineurs [28] and administration of CGRP receptor antagonists can attenuate the pain [29]. CGRP release is reportedly induced by PGE<sub>2</sub>, which is dependent on COX-2 activity in rat TGCs with IL-1 $\beta$  stimulation [17]. Additionally, CCL2 triggers CGRP release in primary nociceptive neurons [30]. In this study, the IL-1 $\beta$ -induced increase in CGRP release was significantly inhibited by parecoxib or bindarit pretreatment in rat TGCs, suggesting that CGRP release depended on COX-2 activity and CCL2 production. These results indicated that ISO reduces neuropathic pain via reduction of CGRP release by inhibiting IL-1 $\beta$ -induced COX-2 activation and CCL2 release in rat TGCs.

In conclusion, the present findings demonstrated that ISO can reduce COX-2 and CCL2 expression and PGE<sub>2</sub>, CCL2, and CGRP production in IL-1 $\beta$ -challenged TGCs. Moreover, the inhibition of IL-1 $\beta$ -induced CGRP release by ISO was dependent on reduction of COX-2 activation and CCL2 release in TGCs. Overall, these results suggested that ISO may be an effective therapeutic agent for alleviating inflammation-induced neuropathic pain.

#### Disclosure of conflict of interest

None.

**Address correspondence to:** Drs. Jianfeng Li and Tieli Dong, Department of Pain Medicine, The Second Affiliated Hospital of Zhengzhou University,

2 Jingba Road, Zhengzhou 450014, Henan, China.  
Tel: 86-371-63974868; Fax: 86-371-63974868;  
E-mail: jianfenglizz@sina.com (JFL); Tel: 86-371-63978600; Fax: 86-371-63978600; E-mail: tiel-dong@sina.com (TLD)

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