Involvement of CCL1/CCR8 in Spinal Cord Dorsal Horn in Remifentanil-Induced Hyperalgesia in Rats

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ABSTRACT

Background: Numerous researches manifested that remifentanil infusion might be responsible for opioid-induced hyperalgesia (OIH), but most studies exploring OIH were hardly designed to clarify underlying mechanisms. Chemokine (C-C motif) ligand 1 (CCL1) was found to be implicated in neuropathic pain, and our previous study has also shown that proinflammatory cytokines have been associated with the induction and maintenance of OIH. However, whether CCL1 could contribute to hyperalgesia induced by remifentanil in rats remains unclear.

Methods: To explore effect of CCL1 on OIH, a neutralizing antibody against CCL1 (anti-CCL1) was administrated intrathecally after remifentanil infusion in rats. Western blotting and immunohistochemistry (IHC) were applied to analyze time course of CCL1 and CCR8 (specific CCL1 receptor) expression in dorsal horn after remifentanil administration. Expression of inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 was evaluated by real-time quantitative polymerase chain reaction (RT-qPCR). Paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) were measured and recorded for 48 post-infusion hours to evaluate mechanical and thermal hyperalgesia.

Results: We discovered that CCL1 and CCR8 expressions in dorsal horn were increased and maintained at a high level from 2 hours to 48 hours, the last examination time, after remifentanil infusion. We found that intrathecal delivery of anti-CCL1 could ameliorate remifentanil related thermal and mechanical hyperalgesia without affecting baseline nociceptive threshold. It was also shown that enhancement of inflammatory mediators (TNF-α, IL-1β, and IL-6) expression in dorsal horn after remifentanil infusion was reversed by anti-CCL1 administration.

Conclusions: Our current results indicated that CCL1 and CCR8 might be implicated in the development of remifentanil-induced hyperalgesia via regulation of cytokines (TNF-α, IL-1β, and IL-6) in dorsal horn in rats.
Remifentanil is a highly potent and ultra-short-acting analgesic of general anesthesia in the clinical setting. However, an increasing number of papers showed that remifentanil at clinically relevant dose (0.1 – 0.5 μg/kg/minute) as an intraoperative analgesic could generate opioid-induced hyperalgesia (OIH), characterized by enhancement of mechanical pronociception and postoperative opioid requirement (1-3). Molecular mechanisms underlying OIH have been considered to be multifactorial and remain elusive (4, 5).

There is no denying the fact that OIH is related to central glutamatergic system and N-methyl-D-aspartate (NMDA) receptor-activation induced central sensitization (6-8). Furthermore, it is well revealed that inflammatory mediators potentiate the glutamatergic synaptic transmission and NMDA receptor NR1 and NR2B subunits activation in the spinal cord during nociceptive responses (9, 10). Our previous study has also indicated that up-regulation of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 in dorsal root ganglion might be implicated in generation and persistence of hyperalgesia caused by remifentanil (11). However, whether and how expression of inflammatory mediators in dorsal horn is elevated in OIH remains unclear.

Although involvement of chemokines in macrophage/monocyte chemotaxis has been well understood, the roles of chemokine in nociceptive process in central nervous system require to be increasingly established. Experimental studies have found that several chemokines might be associated with the induction and maintenance of inflammatory and neuropathic pain (12, 13). Chemokine (C-C motif) ligand 2 (CCL2) has been demonstrated to cause rapid and sustained tactile allodynia and thermal hypernociception through spinal microglia activation (14-16). CCL1 might be one of the crucial mediators in the development of nerve injury-related neuropathic pain, via up-regulation of inflammatory cytokines in spinal cord (17). However, whether CCL1 could induce release of inflammatory cytokines and contribute to OIH has not been reported yet.

This study was performed to test the hypothesis that CCL1 and CCR8 (specific CCL1 receptor) in spinal cord dorsal horn might be involved in OIH through modulation of expression in inflammatory cytokines (TNF-α, IL-1β, and IL-6) in a rat model of remifentanil-induced hypernociception.

**MATERIALS AND METHODS**

**Animals**
Adult male Sprague-Dawley rats (250 g), used throughout all experiments, were obtained from the Laboratory Animal Center of the Military Medical Science Academy of the Chinese People’s Liberation Army and housed under a 12-hour light/dark cycle with food and water available ad libitum. All procedures performed in this study were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University and based on the National Institutes of Health Guide for Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering.

**Drugs Administration**
Rats were anesthetized with sevoflurane (induction, 3.0%; surgery, 1.0%; batch number: 100628; Maruishi Pharmaceutical Co., Osaka, Japan) by a nose mask under sterile conditions. Remifentanil hydrochloride (batch number 090907; Ren-Fu Co., Yichang, China) was dissolved in normal saline (NS) and infused 1 μg/kg/minute for 60 minutes, or NS was infused 0.1 ml/kg/minute for 60 minutes via caudal vein. A neutralizing antibody against CCL1 (anti-CCL1, R&D Systems, Minneapolis, MN, USA) was injected intrathecally in a volume of 5 μl (50 ng) followed by 10 μl NS to flush the catheter, or vehicle (NS) was administrated intrathecally in a volume of 15 μl (50 ng) followed by 10 μl NS to flush the catheter, or vehicle (NS) was administrated intrathecally in a volume of 15 μl after remifentanil or NS infusion. Intrathecal delivery was performed using a microsyringe through an intervertebral space between the L5 and L6 of the spinal cord.

**Nociceptive Behavioral Tests**

**Von Fred Test**
Mechanical hyperalgesia was evaluated using electronic Von Frey filaments (BSEVF3, Harvard Apparatus Co., Holliston, MA, USA) by a previously described method and recorded as the paw withdrawal threshold (PWT) (18). Rats
were housed individually in a cage (20 cm × 20 cm × 20 cm) with a wire mesh bottom (1 cm × 1 cm) and were allowed to acclimatize for 30 minutes to the new environment. Von Frey filaments were applied vertically to the plantar surface of right hind paw and repeated three times at 5-minute interval at each time point. The average value of that filament in grams was considered to be PWT. A maximal cut-off value of 50 g was used to prevent tissue damage. A positive response was defined as complete lifting of the hind paw off the surface of the cage or flinching.

**Hot Plate Test**

Thermal hyperalgesia was determined by hot plate test which was consistent with our previous study (11) and recorded as the paw withdrawal latency (PWL). Rats were placed into a clear plastic chamber on a hot plate (YLS-6B, Huaibei Zhenghua, Biological Instrument Equipment Co., Ltd., Huaibei, China). The hot plate is a round heated surface surrounded by plexiglass and maintained at 52°C. The device is connected to a manually operated timer that records the amount of time the rat spends on the heated surface before showing signs of nociceptive response (clear paw withdrawal, shaking, or licking). Test was repeated three times at 5-minute interval at each time point. A cut-off time of 30 seconds was used to avoid tissue damage to the hind paw. The average of the measurements was used as PWL.

**Western Blot**

The animals were deeply anesthetized with sevoflurane (3%). The L4-L6 segments of spinal cord were isolated rapidly and snap-frozen in liquid nitrogen. The dorsal horn was homogenized in ice-cold lysis buffer containing protease inhibitors (Sigma-Aldrich Co., St. Louis, MO, USA). The lysate was centrifuged and supernatant was removed as the total protein. The loading and blotting of equal amount of total proteins were detected and verified by membrane with monoclonal mouse anti-β-actin antibody (1:5000; Sigma-Aldrich, St. Louis, MO, USA). Samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride (PVDF) membrane. The membranes were blocked incubated overnight at 4°C with polyclonal rabbit antibodies against rat CCL1 or CCR8 (all 1:1000, Abcam, UK), then developed with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (1:2000, Jackson Immuno Research, West Grove, PA, USA) for 1 hour. Membrane bound secondary antibodies were detected using enhanced chemiluminescence solution and visualized using a chemiluminescence imaging system (Syngene, Cambridge, UK). The results were expressed as the percentage of endogenous control (β-actin) immunoreactivity. The density of each specific band was calculated using Gene Tools Match software (Syngene, Cambridge, UK).

**Immunohistochemistry**

The spinal cord samples (L4-L6) were isolated and postfixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 6 hours, and embedded with paraffin. The spinal cords segments were cut into 7 μm-thick sections. After deparaffinization and rehydration, sections were first blocked with goat serum for 1 hour at room temperature. Sections were then incubated over night at 4°C with the primary antibody of rabbit polyclonal CCL1 or CCR8 (1:100; Abcam, UK), followed by biotinylated secondary antibody (1:300, Boster Biological Technology, Ltd., Wuhan, China) for 1 hour at room temperature. Then the sections were incubated with avidin-biotin peroxidase for 20 minutes and detected with diaminobenzidine (DAB substrate kit, Boster Biological Technology Co. Ltd, Wuhan, China). Sections were briefly counterstained with haematoxylin, dehydrated in graded alcohols, cleared in xylene and coverslipped with gum. Images were captured with an Olympus eclipse 80i microscope (Olympus, Tokyo, Japan) for data analysis.

**Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)**

The levels of inflammatory mediators (TNF-α, IL-1β, and IL-6) mRNA in the dorsal horn were determined after the last behavioral testing. Total mRNA was extracted using RNA 4 Aqueous kit (Ambion Inc., Austin, TX, USA). Reverse transcriptase reaction was performed for each mRNA sample using Retroscript kit (Ambion Inc., Austin, TX, USA). 1 mg of total mRNA was used as
template to synthesize first strand cDNA. RT-qPCR was performed with 3 independent repetitions using the Applied Biosystems (ABI Prism) 7900HT Sequence Detection system according to the instruction of SYBER Green PCR Master Mix (Applied Biosystems, Foster city, CA, USA). The reaction program included 2 minutes at 50℃, 10 minutes at 95℃, 40 cycles for 15 seconds at 95℃ and 60 seconds at 60℃. Every gene expression was calculated from the standard curve; quantitative normalization in each sample was performed using the expression of the glyceraldehydes 3-phosphate dehydrogenase (GAPDH) as an internal control using the delta-delta-Ct method (19).

Data were presented as fold change over control. Gene primers sequences were: TNF-α: Forward 5’- CCAGGAGAAAGTCAGCCTCCT- 3’, Reverse 5’-TCAT ACCAGGGCTTGAGCTCA- 3’; IL-1β: Forward 5’- AGAGTGTGGA TCCCAAACAA- 3’, Reverse 5’- AGTCAACTATGTCCCGACCA- 3’; IL-6: Forward 5’- ACACTCCTTAGTCCTCGGCA- 3’, Reverse 5’- CAC GATTTCCCAGAGAA GATGTG- 3’; and GAPDH: Forward 5’- CAC GATTTCCCAGAGAA CATGTG-3’; and GAPDH: Forward 5’- AACAG CAACTCCACTCCTTC- 3’, Reverse 5’- CCTCTTGTGCTAGTGCCT-3’).

Statistical Analysis
All data were expressed as means ± standard deviation (SD). Statistical analysis was computed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The statistical analyses of behavioral testing data were performed by two-way analysis of variance (ANOVA) with repeated measures. The results of Western blot, RT-qPCR and immunohistochemistry were compared using one-way ANOVA. In all cases, P value <0.05 was considered as the criterion for statistical significance.

RESULTS

Time Course of CCL1 and CCR8 Expression in Dorsal Horn after Remifentanil Infusion
Our Western blot analysis showed that compared with NS infusion, CCL1 and CCR8 expressions in dorsal horn significantly increased from 2 hours, peaked at 24 hours and started decreasing from 48 hours (the last examination time) after remifentanil infusion (P<0.01, Figure 1). Peak changes of CCL1 and CCR8 in dorsal horn after remifentanil delivery were also de-
tected and supported by immunohistochemistry staining (Figure 2). Remifentanil caused behaviorally expressed mechanical and thermal hyperalgesia was detectable and maintained from 2 hours to 48 hours after remifentanil delivery (P<0.01, Figure 3). Time course of CCL1 and CCR8 proteins in dorsal horn after remifentanil infusion was well correlated with results of nociceptive behavioral testing. These data indicated that remifentanil infusion could cause a rapid onset (2 hours) and long-lasting (greater than 48 hours) increase in the expressions of CCL1 and CCR8 proteins in spinal cord dorsal horn, and these changes might be involved in hyperalgesic effect of remifentanil.

Pretreatment with Anti-CCL1 could Ameliorate Remifentanil Induced Mechanical and Thermal Hyperalgesia

A neutralizing antibody against CCL1 (anti-CCL1) was intrathecally injected after remifentanil or NS infusion. PWT and PWL were measured at 24 hours before and 2, 6, 24 and 48 hours after remifentanil or NS infusion (Figure 3). Rats treated with remifentanil induced a remarkable decrease in PWT and PWL in comparison with NS infusion (P<0.01). Moreover, rats treated with anti-CCL1 partially and effectively attenuated the decrease of PWT and PWL after remifentanil infusion (P<0.01). However, intrathecal injection of anti-CCL1 after NS infusion had no influence on baseline nociceptive threshold (P>0.05). Together, pretreatment with anti-CCL1 could significantly reverse remifentanil-induced thermal and mechanical hypernociception.

Up-Regulation of Inflammatory Mediators in Dorsal Horn after Remifentanil Infusion was Reversed by Anti-CCL1 Administration

Through the review of literatures on chemokines-cytokines interactions in nociceptive process, we found that changes of cytokines expressions might be measured by RT-qPCR (17, 20, 21). So we performed RT-qPCR to evaluate cytokines levels in our study. As shown in figure 4, we evaluated the gene expressions of inflammatory mediators (TNF-α, IL-1β, and IL-6) in dorsal horn at 24 hours after remifentanil infusion by RT-qPCR. Our current results showed that gene levels of TNF-α, IL-1β, and IL-6 were significantly elevated in dorsal horn in hyperalgesia caused by remifentanil infusion (P<0.01). Moreover, anti-CCL1 intrathecal delivery successfully blocked the increase of TNF-α, IL-1β, and IL-6 gene expression in dorsal horn after remifentanil treatment when compared with remifentanil administration alone (P<0.01). The current results manifested that increase of inflammatory cytokines (TNF-α, IL-1β, and IL-6) in dorsal horn after remifentanil infusion could be significantly reversed by anti-CCL1 treatment, suggesting that CCL1 might be implicated in the gener-
In the present study, we reported that thermal and mechanical hypernociception could be induced by remifentanil infusion, simultaneously, hyperalgesia become detectable and maintained from 2 hours to at least 48 hours. This is the first time to indicate that expression of CCL1 and CCR8 in spinal cord dorsal horn might be rapidly and remarkably increased within 2 hours, lasting for 48 hours after remifentanil administration. Furthermore, it is demonstrated that intrathecal suppression of CCL1/CCR8 could effectively attenuate remifentanil caused pronociception, which may be through down-regulation of inflammatory cytokines (TNF-α, IL-1β, and IL-6) in spinal cord dorsal horn.

According to equivalent dose conversion table between the species, the dose used in rat is 6.25 times of that in human to achieve the same pharmacodynamic effect (22). Simultaneously, the dose of 1.0 μg/kg/minute in rat converting to human dose is 0.16 μg/kg/minute, which is within the clinically accepted doses (1-3). Moreover, the dosage of remifentanil (1.0 μg/kg/minute, 60 minutes) infused in this experiment has been shown to induce hyperalgesia by our previous studies (8, 11, 18, 23). Therefore, we selected and performed a rat model of remifentanil infusion (1 μg/kg/minute for 60 minutes) to investigate opioid-induced pain sensitization to noxious stimuli. Sevoflurane was used for anesthesia because it had no action on baseline nociceptive thresholds (24). In this study, we found that hypernociception occurred from 2 hours and peaked at 24 to 48 hours after remifentanil administration. All data of nociceptive behavioral tests definitely showed that the rat model of remifentanil post-infusion hyperalgesia was established successfully.

Most studies exploring and evaluating OIH are hardly designed to clarify underlying mechanisms (4, 5). It is safe to assert that activation of central NMDA nociceptive system exerts a prime effect on the development and maintenance of OIH (6-8). Gu et al. (25) have provided strong evidence that the expression of NMDA receptor tyrosine phosphorylation in superficial dorsal horn boosts remarkably in the

![Figure 4. Anti-CCL1 Treatment Reduced Expressions of Inflammatory Mediators in Dorsal Horn after Remifentanil Infusion.](image-url)

The L4-L6 segments of dorsal horn were collected for real-time qPCR assay at 24 hours after NS or remifentanil infusion. Values for TNF-α (A), IL-1β (B), and IL-6 (C) mRNA expressions were presented as fold increase over group NS infusion and normalized to the expression of GAPDH. Data were expressed as means ± SD (N=4). #P<0.01 vs group NS infusion, *P<0.01 vs group remifentanil infusion.
maintenance of OIH. Meanwhile, our latest literatures (8, 11, 18, 23) have further disclosed that enhancement of NR2B-containing NMDA receptor expression and trafficking from cytoplasm to surface in spinal cord and dorsal root ganglion might be involved in remifentanil related pain sensitization. It was also found that intraperitoneal delivery of hydrogen-rich saline, an effective anti-inflammatory drug, could successfully block expression and trafficking of NMDA receptor to manage OIH, suggesting the involvement of inflammation in NMDA receptor activation in OIH (11). However, mechanisms underlying NMDA receptor activation were multifactorial and required further research.

Expression and release of inflammatory cytokines in spinal cord have been previously demonstrated to enhance pronociceptive input transmission (26). IL-1β could modulate NR1 phosphorylation and activity to facilitate initiation of pronociception (27). Synaptic efficacy was promoted by TNF-α via modulation of the surface expression in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (28). IL-6 was discovered to be one of the essential mediators of neuropathic and inflammatory pain (29). Spinal IL-17 was known to facilitate and promote inflammatory hyperalgesia through regulation of NMDA receptor phosphorylation (30). In our previous paper, it was convinced that proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in dorsal root ganglion were increased in remifentanil-induced pain sensitization and pretreatment with hydrogen-rich saline could significantly reduce cytokines release to reverse OIH (11). However, whether inflammatory mediators in dorsal horn are involved in hyperalgesia caused by remifentanil remains unclear. As expected, this study found that up-modulation of TNF-α, IL-1β, and IL-6 occurred in spinal cord dorsal horn of remifentanil treated rats, suggesting the involvement of inflammatory cytokines in dorsal horn in remifentanil induced hyperalgesia.

Chemokines and their receptors are highly expressed by neurons, microcytes and astrocytes in the central nervous system, and the glial-neuronal interactions mediated by chemokines and their receptors are increasingly considered to be implicated in the generation and maintenance of pathological pain (12, 13). Combination of fractalkine (CX3CL1) and its receptor could stimulate further release of microglial mediators and sustain the chronic pain state. Furthermore, the interaction among CX3CL1, IL-18 and IL-23 pathway in the spinal cord exerts a crucial effect on the development of sciatic nerve injury induced neuropathic allodynia (31). CCL3/CCL5 signaling is revealed to be one of most critical pathological mediators in central mechanism of spinal nerve injury caused pain facilitation (20). Chemokines and their receptors are highly and nonspecifically generated and expressed by neurons, microcytes and astrocytes. CCL2 is released from primary afferents and acts on CCR2 on microglia (32). CCL2, which can also be expressed and released from astrocytes and act on CCR2 on neurons, might rapidly cause neuropathic pain sensitization by facilitation of excitatory synaptic transmission (14). More importantly, Akimoto et al. (17) have demonstrated that CCL1/CCR8, expressed not only in neurons but also in microcytes and astrocytes, has been discovered and manifested to contribute to neuropathic pain through release of inflammatory regulators and activation of NMDA receptor in spinal cord.

In our current study, we sought to determine whether CCL1/CCR8- cytokines was associated with development of remifentanil induced hypernociception. It is demonstrated, for the first time, that expressions of CCL1 and CCR8 in spinal cord dorsal horn after remifentanil infusion increase within 2 hours, peak at 24 hours and start decreasing from 48 hours, the last examination time. Whether level of CCL1 and CCR8 has a long-term change in remifentanil-induced hyperalgesia still needs to be confirmed. As is acceptable, molecular mechanisms underlying OIH have been considered to be multifactorial and remain elusive (4, 5). Simultaneously, we have pointed out that intrathecal injection of anti-CCL1 could significantly reduce production of TNF-α, IL-1β, and IL-6 in dorsal horn and successfully ameliorate mechanical and thermal hyperalgesia caused by remifentanil, indicating that CCL1/CCR8 signaling might be implicated in development of OIH. But it didn't reverse the increase of PWT, PWL, and cytokines completely, suggesting that other mechanisms might also participate in induction of OIH, despite the criti-
Clinical role of CCL1/CCR8 network in OIH. However, whether NMDA receptor in spinal cord might be activated by hypernociception of CCL1, and whether spinal CCL1 is associated with antihyperalgesic effect of pretreatment with hydrogen-rich saline require further study.

CONCLUSION

In summary, it is conceivable to assert that CCL1/CCR8 might be involved in hyperalgesia induced by remifentanil, through up-regulation of expressions in inflammatory mediators (TNF-α, IL-1β, and IL-6) in spinal cord dorsal horn. Furthermore, pretreatment with intrathecal delivery of anti-CCL1 could effectively and efficiently ameliorate remifentanil-induced mechanical and thermal hyperalgesia. Our current results also suggested that targeting CCL1/CCR8 signal pathway might be a novel therapeutic approach for the treatment of opioid induced hyperalgesia in clinics.

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The authors have declared that no conflict of interest exists.

Guo-Lin Wang and Hai-Yan Wang conceived the experiment; Lin-Lin Zhang, Rui-Chen Shu, Chun-Xuan Wang and Zhi-Fen Wang collected the data; Lin-Lin Zhang, Yong-Hao Yu and Nan Li analyzed the data; Lin-Lin Zhang, Guo-Lin Wang and Rui-Chen Shu wrote the paper.

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