HYDROGEN-RICH SALINE PREVENTS REMIFENTANIL-INDUCED HYPERALGESIA AND INHIBITS MnSOD NITRATION VIA REGULATION OF NR2B-CONTAINING NMDA RECEPTOR IN RATS

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Abstract—Remifentanil administration may subsequently cause paradoxical hyperalgesia in animals and humans, but mechanisms remain unclear. Manganese superoxide dismutase (MnSOD) nitration and inactivation caused by generation of reactive oxygen species and activation of N-methyl-D-aspartate (NMDA) receptors are involved in the induction and maintenance of central neuropathic pain. Hydrogen which selectively removes superoxide has gained much attention in recent years. In this study, we investigated antinociceptive effects of hydrogen-rich saline (HRS) on remifentanil-induced postsurgical hyperalgesia in a rat model of incisional pain. HRS was injected intraperitoneally 10 min before remifentanil infusion (1 µg kg⁻¹ min⁻¹ for 60 min). A selective NR2B antagonist Ro25-6981 was used to investigate whether antihypernociception of HRS is associated with NMDA receptor (NMDAR). Nociception was evaluated by the paw withdrawal mechanical threshold and thermal latency respectively. Then we assessed MnSOD, NR2A and NR2B in spinal cord dorsal horn via Western blot and immunohistochemistry after nociceptive tests. Here, we found that the analgesic effect of remifentanil was followed by long-term hyperalgesia lasting at least postoperative 7 days, which was accompanied with increase in NR2B expression and trafficking from cytoplasm to surface and MnSOD nitration in dorsal horn. Pretreatment with HRS (10 ml/kg) significantly attenuated mechanical and thermal hyperalgesia, blocked NR2B trafficking and MnSOD nitration in dorsal horn after remifentanil infusion. Ro25-6981 not 5 µg but 10 and 50 µg dosage-dependently attenuated hyperalgesia, and inhibited MnSOD nitration. Hyperalgesia and MnSOD nitration were attenuated after the combination of HRS (2.5 ml/kg) and Ro25-6981 (5 µg). In conclusion, HRS (10 ml/kg) might reverse remifentanil-induced hyperalgesia, through regulating NR2B-containing NMDAR trafficking to control MnSOD nitration and enhance MnSOD activity. © 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: hydrogen-rich saline, opioid-induced hyperalgesia, Ro25-6981, N-methyl-o-aspartate, manganese superoxide dismutase.

INTRODUCTION

µ-Opioids have been prevalently engaged in alleviating acute and chronic, non-cancer and cancer-related pain. Unfortunately, opioid administration may be bound up with subsequent opioid-induced hyperalgesia (OIH), even if they initially offer directly analgesic function (Angst and Clark, 2006; Liang et al., 2011; Ohnesorge et al., 2013). OIH is characterized by sensitivity-enhancing to noxious irritation and nociceptive responses to innoxious stimuli. Many proposed molecular mechanisms of OIH, while not yet well understood, have been extensively reviewed (Chu et al., 2008; Lee et al., 2011). There are a dramatically increasing number of basic and clinical studies reporting that administration of the potent, ultra-short-acting opioid remifentanil seems to cause OIH more frequently and predictably compared with others (Celerier et al., 2006; Cabañero et al., 2009; Ishida et al., 2012), likely due to its rapid onset and offset.

N-methyl-D-aspartate (NMDA) receptor, a major subtype of excitatory glutaminergic receptors, exerts an irreplaceable influence on central pain hypersensitivity (Klaus et al., 2004; Chizh, 2007). Functional NMDA receptors are composed of the principal subunit NR1 and one or more modulatory subunits NR2A-D (Mori and Mishina, 1995). Indeed, excitotoxicity is triggered by the selective activation of NR2B-containing NMDA receptors (NMDAR)s (von Engelhardt et al., 2007; Zhao and Joo, 2008; Gu et al., 2009). Our previous studies (Yuan et al., 2013) have further manifested that NMDA receptor NR1 and NR2B subunits membrane trafficking in the spinal cord increased during the development of OIH, mediated by the activation of GSK-3β (glycogen synthase kinase-3β). However, how NR2B-containing NMDAR contributes to OIH remains elusive.

Many reports showed that reactive oxygen species (ROS) were implicated in neuropathic pain, predominantly through spinal mechanisms (Im et al., 2012; Gwak et al., 2013). It is well known that NMDAR...
The experiment segments of the right dorsal horn were collected. Animals with sham operation underwent the surgery, the skin was closed and covered with erythromycin ointment. After hemostasis with gentle pressure, the plantaris muscle, leaving the muscle origin toward the toes of the right hindpaw. Using forceps elevating at 0.5 cm from the edge of the heel and extending 1 cm longitudinal incision was made through the skin, starting 0.1 ml/kg/min−1 for 60 min via caudal vein. A selective NR2B antagonist Ro25-6981 (Sigma–Aldrich Co., St. Louis, MO, USA) was injected intrathecally in a volume of 10 μl followed by 10-μl NS to flush the catheter. Intrathecal injection was made through an intervertebral space at the level of the 5th or 6th lumbar vertebra using a microsyringe, as described by Storkson et al. (1996).

**EXPERIMENTAL PROCEDURES**

**Animals**

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University and were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats (250 g), purchased from the Laboratory Animal Center of the Military Medical Science Academy of the Chinese People’s Liberation Army, were used throughout all experiments and housed with a reverse light/dark cycle of 12/12 h, and fed ad libitum. Every effort was made to minimize animal suffering.

**Surgery**

The incisional postoperative pain model of rats followed the procedures described by Brennan et al. (1996). Rats were anesthetized with sevoflurane (induction, 3.0%; surgery, 1.5%) by a nose mask under sterile conditions. A 1-cm longitudinal incision was made through the skin, starting at 0.5 cm from the edge of the heel and extending toward the toes of the right hindpaw. Using forceps elevated the plantaris muscle, leaving the muscle origin and insertion intact. After hemostasis with gentle pressure, the skin was closed and covered with erythromycin ointment. Animals with sham operation underwent the same procedure without incision.

**Drugs**

HRS was prepared as described by Li et al. (2010). Hydrogen was dissolved in normal saline (NS) for 6 h under high pressure (0.4 MPa) to a supersaturated level using a hydrogen-rich water producing apparatus (YUTAKA Engineering Co., Tokyo, Japan). The saturated hydrogen saline was stored under atmospheric pressure at 4 °C in an aluminum bag, sterilized by gamma radiation and freshly prepared every week to ensure a constant concentration more than 0.6 mmol/L.

Sevoflurane (batch number: 100628; Maruishi Pharmaceutical Co., Osaka, Japan). Remifentanil hydrochloride (batch number 090907; RenFu Co., Yichang, China) was dissolved in NS and infused 1 μg kg−1 min−1 for 60 min, and NS was infused 0.1 ml/kg−1 min−1 for 60 min via caudal vein. A selective NR2B antagonist Ro25-6981 (Sigma–Aldrich Co., St. Louis, MO, USA) was injected intrathecally in a volume of 10 μl followed by 10-μl NS to flush the catheter. Intrathecal injection was made through an intervertebral space at the level of the 5th or 6th lumbar vertebra using a microsyringe.

**Experimental protocol**

**Experiment 1: changes in mechanical and thermal hyperalgesia induced by remifentanil, MnSOD expression and nitration, and NMDAR expression and trafficking after pretreatment with HRS.** The experiment was randomly divided into eight groups with eight rats in each group: C group (a sham operation, NS i.v.); I group (NS i.v.); G group (glycine 15 μg kg−1 min−1 for 60 min, i.v.); Glycine is an accessory in the pharmaceutical preparation of remifentanil); R group (remifentanil i.v.); H + C group (HRS i.p., a sham operation, NS i.v.); H + I group (HRS i.p., NS i.v.); H + G group (HRS i.p., glycine i.v.); H + R group (HRS i.p., remifentanil i.v.). HRS (10 ml/kg) was injected 10 min before NS, glycine and remifentanil infusion. A surgical incision was prepared in all groups except group C and H + C. Paw withdrawal mechanical threshold (PWT) and paw withdrawal thermal latency (PWL) were measured at 1 day before and 1, 2, 3, 5, 7 days after operation. The L₄–L₆ segments of the right dorsal horn were collected after the last behavioral testing for determining the expression and nitration of MnSOD, and the expression of the total and membrane NMDAR (NR2A and NR2B).

**Experiment 2: role of NMDA receptor in MnSOD nitration and antihyperalgesia of HRS.** The experiment was divided into eight groups with 8 rats in each group: group C (NS i.v.); group C + Ro 3 (Ro 25-6981 50 μg); group R; group Ro 1, Ro 2, Ro 3 (Ro 25-6981 5 μg, 10 μg, 50 μg); group H; group Ro 1 + H. A surgical incision was prepared in all groups except group C and C + Ro 3. Ro25-6981 was given intrathecally after NS or remifentanil infusion. HRS (2.5 ml/kg) was injected intraperitoneally 10 min before remifentanil infusion. PWT and PWL were measured as Experiment 1. The L₄–L₆ segments of the right dorsal horn were collected after completing behavioral tests for determining MnSOD expression and nitration.

**Nociceptive behavioral testing**

To evaluate mechanical hyperalgesia, PWT was measured by electronic Von Frey filaments (BSEVF3, Intraplantar injection of 3 μl of 0.75% formalin (Sigma–Aldrich Co., St. Louis, MO, USA) was injected into the right hindpaw of rats. The formalin injection site was marked with a micropen. A 3 mm circular wound was made around the injection site using a sterile scalpel. The formalin was injected using a 30-gauge needle. The formalin solution was removed after 2 min. The formalin solution was removed after 2 min. The 2 min mark was used to determine the time when mechanical hyperalgesia occurred. The formalin solution was removed after 2 min.

**Drugs**

Remifentanil hydrochloride (batch number 090907; RenFu Co., Yichang, China) was dissolved in NS and infused 1 μg kg−1 min−1 for 60 min, and NS was infused 0.1 ml/kg−1 min−1 for 60 min via caudal vein. A selective NR2B antagonist Ro25-6981 (Sigma–Aldrich Co., St. Louis, MO, USA) was injected intrathecally in a volume of 10 μl followed by 10-μl NS to flush the catheter. Intrathecal injection was made through an intervertebral space at the level of the 5th or 6th lumbar vertebra using a microsyringe, as described by Storkson et al. (1996).
Harvard Apparatus Co., Holliston, MA, USA). Rats were placed individually in a cage (20 cm x 20 cm x 20 cm) with a wire mesh bottom (1 cm x 1 cm) and allowed to acclimatize for 30 min. Von Frey filaments were applied vertically to the plantar side of the right hind paw and repeated three times at 10-min interval at each time point. A positive response was defined as complete lifting of the hind paw off the surface of the cage or flinching. A maximal cut-off value of 50 g was used to prevent tissue damage.

To evaluate thermal hyperalgesia, rats were placed into a clear plastic chamber on a hot plate (YLS-6B, Zhenghua Biological Instrument Equipment Co., Huaibei, Anhui, 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 nociception. Clear paw withdrawal, shaking, and/or licking were considered as the percentage of endogenous control (Li et al., 2013).

Western blot
The animals were anesthetized with sevoflurane (3%). The L₄–L₆ spinal cord segments were removed rapidly and snap-frozen in liquid nitrogen after all behavioral tests. The right dorsal horn was homogenized in ice-cold sodium dodecyl sulfate sample buffer containing protease inhibitors (Sigma–Aldrich Co., St. Louis, MO, USA). The lysate was centrifuged and supernatant was removed as the total protein. A membrane compartment protein extraction kit (Biochain Institute, Inc., Hayward, CA, USA) was used to extract the membrane fraction of the dorsal horn. The protein content was determined using the bicinechonic acid assay method. The loading and blotting of equal amounts of membrane and total proteins were detected and verified by membrane with mouse anti-rat epidermal growth factor receptor (EGFR, 1:2,000; MBL, Naka-ku Nagoya, Japan) and monoclonal mouse anti-β-actin antibody (1:5000; Sigma–Aldrich, St. Louis, MO, USA), respectively. Samples were separated on 10% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and transferred onto nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in Tris-Tween saline for 1 h and incubated overnight at 4 °C with polyclonal rabbit antibodies against rat NR2A, NR2B, MnSOD or anti-nitrotyrosine (all 1:1000, Abcam, Cambridge, UK), followed by biotinylated secondary antibody (1:300, Boster Biological Technology, Ltd., Wuhan, China) for 45 min at room temperature. Then the sections were incubated with avidin–biotin peroxidase for 20 min and detected with diaminobenzidine (DAB substrate kit, Boster Biological Technology). Sections were briefly counterstained with hematoxylin, dehydrated in graded alcohols, cleared in xylene and coverslipped with gum. Images were acquired with an Olympus eclipse 80i microscope (Olympus, Tokyo, Japan).

Immunohistochemistry
The L₄–L₆ spinal cords were removed after the last behavioral testing, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) (PH 7.4) for 6 h, and embedded with paraffin. The spinal cords segments were cut into 7-µm-thick sections. After deparaffinization and rehydration, sections were treated with PBS containing 5% normal goat serum for 30 min at room temperature to block non-specific reactions. Sections were incubated overnight at 4 °C with the primary antibody of rabbit polyclonal anti-NR2B (1:100; Abbcam, Cambridge, UK), followed by biotinylated secondary antibody (1:300, Boster Biological Technology, Ltd., Wuhan, China) for 45 min at room temperature. Then the sections were incubated with avidin–biotin peroxidase for 20 min and detected with diaminobenzidine (DAB substrate kit, Boster Biological Technology). Sections were briefly counterstained with hematoxylin, dehydrated in graded alcohols, cleared in xylene and coverslipped with gum. Images were acquired with an Olympus eclipse 80i microscope (Olympus, Tokyo, Japan).

Statistical analysis
Statistical analysis was performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Statistical analyses of behavioral testing data were performed using a two-way analysis of variance (ANOVA) with repeated measures. The results of Western blot and immunohistochemistry were analyzed by a one-way ANOVA. A P value < 0.05 was considered statistically significant. Data of all experiments were expressed as mean ± SD.

RESULTS
Pretreatment with HRS could attenuate postoperative mechanical and thermal hyperalgesia induced by remifentanil
Compared with group C, PWT and PWL were significantly decreased in group I and G at 1, 2, 3 days after infusion (P < 0.01), moreover, there is no difference between group I and G (P > 0.05). These data suggested that mechanical and thermal hyperalgesia were caused by incision not glycine infusion (15 µg kg⁻¹ min⁻¹) and continued for less than 5 days. Compared with group C, I and G, group R significantly aggravated and prolonged mechanical and thermal hyperalgesia induced by the incision, which was indicated by a significant decrease in PWT and PWL (P < 0.01). Only remifentanil-induced hyperalgesia lasted up to more than 7 days in that PWT and PWL were still significantly decreased in group R (P < 0.01) on the 7th day after operation. HRS delivery had no influence on baseline nociceptive threshold (P > 0.05). Compared with group I and R, group H + I and H + R induced a remarkable increase in PWT and PWL (P < 0.01), which manifested HRS (10 ml/kg) might play a preventive role in hyperalgesia caused by incision and remifentanil (Figs. 1 and 2).
Fig. 1. Antinociceptive effect of hydrogen-rich saline (HRS) on mechanical postoperative hyperalgesia induced by remifentanil. Pretreatment with HRS (10 ml/kg) was injected intraperitoneally. Paw withdrawal mechanical threshold (PWT) was measured on the right hind paw and recorded at 1 days before and 1, 2, 3, 5 and 7 days after operation. Data were expressed as mean ± SD (n = 8). #P < 0.01 vs group C, $P < 0.01 vs group I, &P < 0.01 vs group G, ⁄P < 0.01 vs group R.

Fig. 2. Antinociceptive effect of hydrogen-rich saline (HRS) on thermal postoperative hyperalgesia induced by remifentanil. Pretreatment with HRS (10 ml/kg) was injected intraperitoneally. Paw withdrawal thermal latency (PWL) was measured and recorded at 1 days before and 1, 2, 3, 5 and 7 days after operation. Data were expressed as mean ± SD (n = 8). #P < 0.01 vs group C, $P < 0.01 vs group I, &P < 0.01 vs group G, ⁄P < 0.01 vs group R.
Increase of MnSOD nitration in dorsal horn after remifentanil infusion was reversed by HRS administration

Compared with group C, MnSOD nitration in dorsal horn was significantly intensified in Group R ($P < 0.01$). Administration of HRS caused a significant decrease in the level of MnSOD nitration in comparison with group R ($P < 0.01$). However, there are no significant changes in the level of MnSOD protein in all groups ($P > 0.05$). Together, these data showed that remifentanil might aggravate surgery-related hyperalgesia via enhancement of MnSOD nitration but not affecting MnSOD expression; furthermore, antihyperalgesic effect of HRS was associated with decrease in MnSOD nitration in the spinal cord dorsal horn (Fig. 3).

Increase of NR2B-containing NMDA receptor expression and trafficking in dorsal horn after remifentanil infusion was reversed by HRS administration

Compared with group C, I and G, the total (t) and membrane (m) levels of NR2B were elevated in group R ($P < 0.01$, Fig. 4); NR2B membrane trafficking from cytoplasm to surface was raised, which was indicated by a significant increase in the ratio of mNR2B/tNR2B after remifentanil infusion ($P < 0.01$); compared with group R, administration of HRS caused a significant decrease in the expression of the total and membrane NR2B ($P < 0.01$), NR2B membrane trafficking from cytoplasm to surface was suppressed after HRS administration, which was observed by a significant decrease in the ratio of mNR2B/tNR2B ($P < 0.01$). Changes of NR2B expression in dorsal horn after remifentanil and HRS administration were also detected and supported by immunohistochemistry staining (Fig. 5). No significant alteration in the expression and trafficking of NR2A was seen in all groups ($P > 0.05$, Fig. 4). These data suggested that HRS could successfully interdict NMDAR expression and membrane trafficking from cytoplasm to surface in the dorsal horn after remifentanil infusion, more precisely, NR2B-containing NMDAR.

Ro25-6981 might attenuate hyperalgesia through controlling MnSOD nitration in dorsal horn

Intrathecal injection of Ro25-6981 had no effect on baseline nociceptive threshold ($P > 0.05$, Fig. 6A, B).
Fig. 5. Effect of hydrogen-rich saline (HRS) on expression of NR2B-containing N-methyl-D-aspartate (NMDA) receptor by immunohistochemistry staining. The L4–L6 spinal cords were removed after the last behavioral testing for Immunohistochemistry. When compared with group C, NR2B expression in dorsal horn dramatically increased after remifentanil infusion; the increase of NR2B expression induced by remifentanil was significantly suppressed by pretreatment with HRS (10 ml/kg). Representative photomicrographs of the right dorsal horn of the L4–6 spinal cord are shown here (Scale bar = 50 μm). Data were expressed as mean ± SD (n = 4). \*P < 0.01 vs group C, \#P < 0.01 vs group R.

Fig. 6. Dose-dependently antinociceptive effect of Ro25-6981 on hyperalgesia induced by remifentanil via manganese superoxide dismutase (MnSOD). A selective NR2B antagonist Ro25-6981 (5 μg, 10 and 50 μg) was injected intrathecally after NS or remifentanil infusion. Paw withdrawal mechanical threshold (PWT, A) and paw withdrawal thermal latency (PWL, B) were evaluated at 1 days before and 1, 2, 3, 5 and 7 days after operation. Data were expressed as mean ± SD (n = 8). The right dorsal horn L4–L6 segments were collected after the last behavioral testing for Western blot assay. (C) Bands of Western blot for the expression and nitration of MnSOD protein. β-actin was the internal standard. (D) Values for the ratios of MnSOD/β-actin, nitrated MnSOD/β-actin and nitrated MnSOD/MnSOD are normalized to C group. Data were expressed as mean ± SD (n = 4). \#P < 0.01 vs baseline, \*P < 0.01 vs group C, \#P < 0.01 vs group R, \#P < 0.01 vs group Ro2.
There are no significant differences between group R and Ro1 ($P > 0.05$), while compared with group R, group Ro2 and Ro3 induced a remarkable and dose-dependent increase in PWT and PWL ($P < 0.01$, Fig. 6A, B).

Compared with group R, administration of Ro25-6981 not 5 µg but 10 and 50 µg dosage-dependently inhibited MnSOD nitration, which was indicated by a significant decrease in the level of nitrated MnSOD and the ratio of nitrated MnSOD/MnSOD ($P < 0.01$, Fig. 6C, D). However, no significant alternation of MnSOD protein expressions in dorsal horn was observed in all groups ($P > 0.05$, Fig. 6C, D).

These detailed results suggested that NR2B-containing NMDAR might participate in increase of MnSOD nitration in OIH, furthermore, inhibition of NR2B with Ro25-6981 not 5 µg but 10 and 50 µg dosage-dependently attenuated remifentanil-induced hyperalgesia, likely through controlling MnSOD nitration in dorsal horn.

**NR2B-containing NMDA receptor was involved in antihyperalgesic effect of HRS via inhibition of MnSOD nitration in dorsal horn**

Behavioral tests (Fig. 7A, B) revealed that HRS (2.5 mg/kg), and Ro25-6981 (5 µg) with subthreshold doses did not alter PWT and PWL alone ($P > 0.05$), but mechanical and thermal hyperalgesia were remarkably improved by the combination of HRS and Ro25-6981 ($P < 0.01$).

Increase of MnSOD nitration in dorsal horn after remifentanil infusion was reduced by pre-administration of HRS and Ro25-6981 with subthreshold doses ($P < 0.01$, Fig. 7C, D). However, there are no significant changes in the expression of MnSOD in all groups ($P > 0.05$, Fig. 7C, D). Together, these data suggested that NR2B-containing NMDAR was involved in antihyperalgesic effect of HRS via incompletely blocking MnSOD nitration without affecting MnSOD expression in dorsal horn.

**DISCUSSION**

In the current study, it was demonstrated that the mechanical and thermal hyperalgesia caused by incision could be significantly aggravated and prolonged by remifentanil but not glycine infusion, meanwhile, remifentanil induced pronociception became observed on postsurgical 1 day and continued to at least 7 days. Inhibition of NR2B with Ro25-6981 not 5 µg but 10 and 50 µg dosage-dependently attenuated remifentanil-induced hyperalgesia, likely through controlling MnSOD nitration.
nitrination in dorsal horn. More importantly, HRS definitely exerted an effectively preventive effect on hyperalgesia, which might be via inhibition of NR2B-containing NMDAR expression and trafficking from cytoplasm to surface, thus contributing to the decrease of MnSOD nitrination and increase of MnSOD activity in the spinal cord dorsal horn.

Our previous study has disclosed that administration of remifentanil in sham or naïve animals could induce hyperalgesia (Li et al., 2013). We selected and designed the model of incision pain which has been generally used in studies of opioid-induced postoperative hyperalgesia to explore whether the intraoperative medication of opioids would alter the nociception to surgery and aggravate incision-related hyperalgesia at this time. Sevoflurane was selected for anesthesia in that it was previously revealed without influence on pronociceptive thresholds (Celerier et al., 2006). Remifentanil at a rate of 1 μg kg⁻¹ min⁻¹ for 60 min used in this study depended predominantly on clinical practice and has been shown to induce hyperalgesia by our previous studies (Li et al., 2013; Yuan et al., 2013). Our previous studies have discovered that OIH starts from 2 h and reaches its peak at 24–48 h after the operation. It is not well known whether opioids could generate long-term hyperalgesia, therefore, we conducted nociceptive behavioral tests 1 day before and 1, 2, 3, 5, 7 days after the surgery. It is conceivable to assert with behavioral tests that we succeeded in establishing the rat model of postsurgical pain sensitization induced by remifentanil.

Enhancements of expression and function of NMDA receptor (Zhao and Joo, 2008), especially NR2B-containing NMDAR, in the spinal cord are considered being indispensable to the nociceptive transmission and the development of OIH (Gu et al., 2009; Jiang et al., 2013). A recent study has reported that remifentanil could increase the amplitudes of NMDA-mediated miniature excitatory postsynaptic current (mEPSC) and decrease the interevent intervals of NMDA-mediated mEPSC to enhance NMDAR function, leading to hyperalgesia (Li et al., 2013). In our present study, we discovered that only remifentanil could potentiate the total, membrane expressions and membrane trafficking of NR2B, without any effect on NR2A.

Glycine, as an adjunct of remifentanil injection in clinical preparation, is an obligatory NMDA receptor coagonist (Guntz et al., 2005). During acute pain, glycine in spinal cord interneurons causes NMDAR facilitation and induces the development of chronic pain (Ahmadi et al., 2003). To test whether glycine could cause NMDAR activation and modify the nociceptive threshold in our model, glycine infusion group was needed. However, our results showed that glycine infusion (15-μg kg⁻¹ min⁻¹) seemed to have no action on expression and trafficking of NMDAR and pronociceptive threshold, probably due to the selected low dose based on containment of remifentanil injection (Kostera-Pruszczczyk et al., 2002).

Several literatures have pointed out that ROS and MnSOD nitrination were implicated in neuropathic pain (Yowtak et al., 2011; Gwak et al., 2013). Hydrogen could selectively remove superoxide and heighten MnSOD activity to exert therapeutic antioxidant function (Zhou et al., 2013). HRS is the first time to be engaged in the model of remifentanil-induced postsurgical hyperalgesia. Moreover, we found and confirmed that HRS could successfully alleviate mechanical and thermal hyperalgesia induced by opioids, possibly via inhibition of MnSOD nitrination and enhancement of MnSOD activity. We also discovered that antihyperalgesic effect of HRS (10 ml/kg) might last for at least 7 days (at last examination day), likely due to the high dose and inhibiting generation and maintenance of hyperalgesia in the early phase. Our previous study (Zhang et al., 2014) has manifested that HRS not 2.5 ml/kg but 5 and 10 ml/kg could dose-dependently produce a rapid-onset (2 h, as early as generation of OIH) increase in PWT and PWL after remifentanil infusion. In addition, peak concentration in arterial blood after HRS 10 ml/kg is almost 30 μmol/L and threefold greater than minimal effective concentration. So we supposed that HRS (10 ml/kg) might suppress production of hyperalgesia to relieve long-lasting persistence of hyperalgesia. The current evidences continue to be insufficient to explain this result, further researches are thus needed to explore mechanisms.

Although the specific mechanisms of OIH are complicated and unclear, significant proposed mechanisms are the central glutaminergic system, spinal dynorphins, descending facilitation, and genetic mechanisms. Of these, the central glutaminergic system is considered the most common possibility (Chu et al., 2008; Lee et al., 2011). However, how NMDAR activation contributes to OIH remains elusive. Accumulating evidence showed that NMDAR activation could give rise to MnSOD nitrination and inactivation, thus producing hyperalgesia (Chen et al., 2010). However, there is no report which NMDAR subtype is the key. In our present study, we found that a NR2B-selective antagonist Ro25-6981 not 5 μg but 10 and 50 μg dosage-dependently attenuated remifentanil-induced hyperalgesia, likely through controlling MnSOD nitrination in dorsal horn. This is the first report that NMDAR NR2B subunit played a cardinal role in the nitrination and inactivation of MnSOD after remifentanil infusion, suggesting that inhibitory effect of HRS on MnSOD nitrination might be associated with NR2B-containing NMDAR. These data also hinted that NR2B was a pivotal and valid target for prevention of OIH (Jiang et al., 2013).

To investigate whether antihyperalgesic effect of HRS is associated with NMDA receptor, on the one hand, we used a selective NR2B antagonist Ro25-6981 with subthreshold dose. Concurrently, our results showed that antinociception and suppression of MnSOD nitrination in dorsal horn produced by HRS were induced and generated by co-administration of Ro25-6981, while both alone in subthreshold doses did not display any functions. On the other hand, administration of HRS caused a significant decrease in the expression and trafficking of NR2B in dorsal horn. Taken together, these data indicated that HRS could successfully reverse remifentanil-induced hyperalgesia through interdicting membrane trafficking of NR2B-containing NMDAR to control MnSOD nitrination in the dorsal horn.
This is the first time that NR2B-containing NMDAR is involved in the antioxidant mechanism of HRS. The specific mechanism by which HRS regulates NMDA receptors trafficking in dorsal horn remains equivocal and needs to be comprehended and clarified in future studies.

Abundant articles have demonstrated that remifentanil at clinically relevant dose (0.1–0.5 μg/kg/min) as an intraoperative analgesic could cause mechanical pain sensitivity and enhance postoperative opioid consumption (Bormann-Cimenti et al., 2012). Therefore, preemptive analgesia for remifentanil-induced hyperalgesia is dramatically indispensable to postoperative comfort and satisfaction. Multifarious strategies have been applied to attenuating, reversing, and managing OIH (Lee et al., 2011). Early postoperative pain and the quantity of postoperative opioids demand could be restrained by administration of NMDAR inhibitor (Hong et al., 2011), COX-2 inhibitor (Lenz et al., 2011), α2-adrenergic receptor agonist (Zheng et al., 2012), and antiepileptic drugs (Bannister et al., 2011). However, these drugs have side effects of a different degree. It is conceivable that HRS which has the advantages of cheap, convenient and no side effect for use may be beneficial in the pre-emptive treatment of OIH.

CONCLUSION

In summary, pretreatment with intraperitoneal administration of HRS (10 ml/kg) could effectively reverse postsurgical hyperalgesia induced by remifentanil, probably through blocking NR2B-containing NMDA receptor expression and membrane trafficking from cytoplasm to surface to control MnSOD nitration and enhance MnSOD activity in the spinal cord dorsal horn. Our present results suggested that HRS and a selective NR2B antagonist Ro25-6981 might be effective and efficient novel options for the treatment of remifentanil-induced postoperative hyperalgesia and other neuropathic pain in clinics.

AUTHOR CONTRIBUTIONS

GL Wang and HY. Wang conceived the experiment; LL Zhang, RC Shu, CY Wang and MM. Wang collected the data; LL Zhang, YH. Yu and MH. Yang analyzed the data; LL Zhang, GL Wang and RC Shu wrote the paper.

CONFLICT OF INTEREST STATEMENT

The authors have declared that no conflict of interest exists.

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