Spinal Peroxynitrite Contributes to Remifentanil-induced Postoperative Hyperalgesia *via* Enhancement of Divalent Metal Transporter 1 without Iron-responsive Element–mediated Iron Accumulation in Rats

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ABSTRACT

Background: Hyperalgesia is one of the negative consequences following intraoperative analgesia with remifentanil. Peroxynitrite is a critical determinant in nociceptive process. Peroxynitrite inactivates iron-sulfur cluster that results in mitochondrial dysfunction and the release of iron, leading to mitochondrial iron accumulation. Iron accumulation mediated by divalent metal transporter 1 (DMT1) plays a key role in *N*-methyl-D-aspartate neurotoxicity. This study aims to determine whether peroxynitrite contributes to remifentanil-induced postoperative hyperalgesia *via* DMT1-mediated iron accumulation.

Methods: Behavior testing was performed in rat model at different time points. Three-nitrotyrosine, nitrated manganese superoxide dismutase, and DMT1 with/without iron-responsive element [DMT1(+)IRE and DMT1(-)IRE] in spinal cord were detected by Western blot and immunohistochemistry. Spinal iron concentration was measured using the Perl stain and atomic absorption spectrophotometer. Hydrogen-rich saline imparting selectivity for peroxynitrite decomposition and iron chelator was applied in mechanistic study on the roles of peroxynitrite and iron, as well as the prevention of hyperalgesia.

Results: Remifentanil induced thermal and mechanical hyperalgesia at postoperative 48 h. Compared with control, there were higher levels of 3-nitrotyrosine (mean ± SD, hyperalgesia *vs.* control, 1.22 ± 0.18 *vs.* 0.25 ± 0.05, n = 4), nitrated manganese superoxide dismutase (1.01 ± 0.1 *vs.* 0.19 ± 0.03, n = 4), DMT1(-)IRE (1.42 ± 0.19 *vs.* 0.33 ± 0.06, n = 4), and iron concentration (12.87 ± 1.14 *vs.* 5.26 ± 0.61 μg/g, n = 6) in remifentanil-induced postoperative hyperalgesia, while DMT1(+)IRE was unaffected. Eliminating peroxynitrite with hydrogen-rich saline protected against hyperalgesia and attenuated DMT1(-)IRE overexpression and iron accumulation. Iron chelator prevented hyperalgesia in a dose-dependent manner.

Conclusions: Our study identifies that spinal peroxynitrite activates DMT1(-)IRE, leading to abnormal iron accumulation in remifentanil-induced postoperative hyperalgesia, while providing the rationale for the development of molecular hydrogen and "iron-targeted" therapies. **(ANESTHESIOLOGY 2015; 122:908-20)**

REMIFENTANIL is a potent, short-acting opioid, which can be a component of balanced anesthesia. Remifentanil is capable of inducing hyperalgesia,[1–5] while its clinical significance and effective prevention strategy are still unclear.[6,7] Studies on the mechanisms of remifentanil-induced hyperalgesia need to be completed to investigate specific interventions that could curtail the risk of development in populations at the highest risk.

Peroxynitrite, the product of the interaction between superoxide and nitric oxide,[8] acts as a potent nitroxidative species and a significant signaling molecule in the development of peripheral and central sensitization during pain.[9–11] The critical pronociceptive role of peroxynitrite is associated with mitochondrial dysfunction triggered by nitration of manganese superoxide dismutase (MnSOD) in neuropathic and inflammatory pain.[12–15] In addition, enhanced glutamatergic signaling and neuroinflammation in spinal cord are involved in the mechanisms by which peroxynitrite contributes to nociceptive processing.[12,16,17] Recently, peroxynitrite is suggested to be selectively eliminated by hydrogen, a novel therapeutic antioxidant, without perturbing normal cellular functions,[18,19] and this notion is supported by both basic and clinical data of various diseases.[20–23] Despite these established effects, the role of peroxynitrite in remifentanil-induced postoperative hyperalgesia and conceivable mechanism is unclear.

What We Already Know about This Topic

- The intraoperative use of remifentanil is associated with opioid-induced hyperalgesia
- Peroxynitrite is an oxidative molecule previously associated with hyperalgesia in models of neuropathic and inflammatory pain

What This Article Tells Us That Is New

- Spinal cord levels of 3-nitrotyrosine, a biomarker for peroxynitrite production, were elevated after remifentanil infusion in rats
- The administration of hydrogen-rich saline both reduced peroxynitrite production and reduced hyperalgesia after remifentanil infusion

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 Anesthesiology 2015; 122:908-20

Divalent metal transporter 1 (DMT1) localized to plasma membrane is composed of 12 transmembrane domains and is the main import channel for iron.[24] There are four DMT1 isoforms that arose from messenger RNA (mRNA) transcription that vary at mRNA 5′ ends (starting in exon 1A or exon 1B) and at mRNA 3′ ends generating isoforms with or without the iron-responsive element (IRE), named divalent metal transporter 1 without iron-responsive element [DMT1(-)IRE] and divalent metal transporter 1 with iron-responsive element [DMT1(+)IRE].[25] DMT1(-)IRE has been identified as a vital target responsible for increased iron uptake under several pathologic conditions.[26–28] DMT1-mediated iron overload is suggested to participate in *N*-methyl-D-aspartate (NMDA) neurotoxicity that is prevented by selective iron chelation.[29] The activation of NMDA receptor is a key determinant of central sensitization relevant to the development of opioid-induced hyperalgesia.[30] Iron is essential for life, while it produces reactive oxygen species and causes cell damage if in excess. Furthermore, the association among inflammatory damage, aberrant iron accumulation, and oxidative stress has been established in enormous pathologic conditions such as diabetes, cancer, and neurodegenerations.[31–34]

The purposes of this study were to affirm whether (1) peroxynitrite contributes to remifentanil-induced postoperative hyperalgesia, and removing peroxynitrite with hydrogen-rich saline is a beneficial approach to prevent hyperalgesia; meanwhile, whether (2) DMT1-mediated iron accumulation is associated with remifentanil-induced postoperative hyperalgesia; and, if so, whether (3) there is relationship between peroxynitrite formation and DMT1-mediated iron accumulation in this setting.

Materials and Methods

Animal Preparation

All experiments were approved by the Scientific and Ethics Committee of Tianjin Medical University (Tianjin, China) and adhered to the guidelines of the National Institutes of Health for the use of experimental animals. Mature male Sprague-Dawley rats (240 to 260 g) were purchased from the Military Medical Science Academy Laboratory (Beijing, China). Rats were housed four per cage and raised in a controlled environment (12-h light/dark cycle, room temperature 22 ± 2°C, 60 to 80% relative humidity) with food and water available *ad libitum*. Animals were randomized, and experimenters were blinded to treatment conditions in the experiments explained below.

Surgical Procedure

We used the incisional postoperative pain model confirmed in our laboratory.[35] Briefly, rats were anesthetized with sevoflurane (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) for 60 min (induction, 3.0% v/v; surgery, 1.0 % v/v) using a nose mask. A 1.0-cm longitudinal incision was made through the skin and fascia of the plantar surface of right hind paw, starting 0.5 cm away from the edge of the heel and extending toward the toes. The plantaris muscle was then exposed and incised longitudinally, maintaining the muscle origin and inserting integrally. The skin was closed with 4-0 silk suture after hemostasis; finally, the wound was covered with erythromycin ointment. Control rats with a sham procedure underwent the same exposure of anesthesia for 60 min, without incision.

Drugs

Intravenous infusion of remifentanil (Yichang Renfu Pharmaceutical Co., Yichang, China) was administrated at the rate of 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 60 min in this experiment, and the dose of remifentanil which can induce hyperalgesia was investigated in our previous study.[36] Saline (0.1 ml $\cdot kg^{-1} \cdot min^{-1}$, 60 min, IV) was used as control treatment. Hydrogen-rich saline was freshly prepared, as described by Li *et al.*[22] Hydrogen was dissolved in saline for 6 h under high pressure (0.4 MPa) to a supersaturated level (≥0.6 mmol/l) using a hydrogen-rich water producing apparatus. Intraperitoneal injection of hydrogen-rich saline (10 ml/kg) was applied at 10 min before surgery. Iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) was delivered by intrathecal injection using the lower lumbar approach, as described previously.[37] SIH (1, 5, and 10 μg) was injected with a total volume of 10 μl followed by 10 μl saline to flush the intrathecal catheter and was given at 10 min before surgery. A schematic view of the experimental design is shown in figure 1.

Behavior Testing

Mechanical Hyperalgesia. Rats were placed in elevated wire chambers (20 × 20 × 20 cm) with a mesh bottom and acclimated for 1 h before tests to achieve immobility. Mechanical hyperalgesia was measured using the electronic von Frey filament (BSEVF3; Harvard Apparatus, Holliston, MA),[36] which exerts a linear increasing mechanical pressure to the dorsum bordering the incision of the rat's right hind paw. The paw withdrawal threshold (PWT) was defined as the pressure (g) at which the rat withdrew, shook, or licked its paw. To avert tissue damage, a cutoff threshold was set at 50 g. The test was repeated three times with an interval of 5 min, and then the mean PWT was evaluated from the average value of the three trials.

Thermal Hyperalgesia. Rats were habituated to the test environment for 1 h before any examination. Thermal hyperalgesia was measured with the intelligent hotplate equipment (YLS-6B; Zhenghua Biological Instrument Equipment Co., Ltd., Huibei, China), as previously described.[35] Rats were placed on the hotplate (50°C) until a positive response occurred, which was defined as a clear paw withdrawal. The time appearing positive response was recorded as the paw withdrawal latency (PWL). Also, the test was repeated three

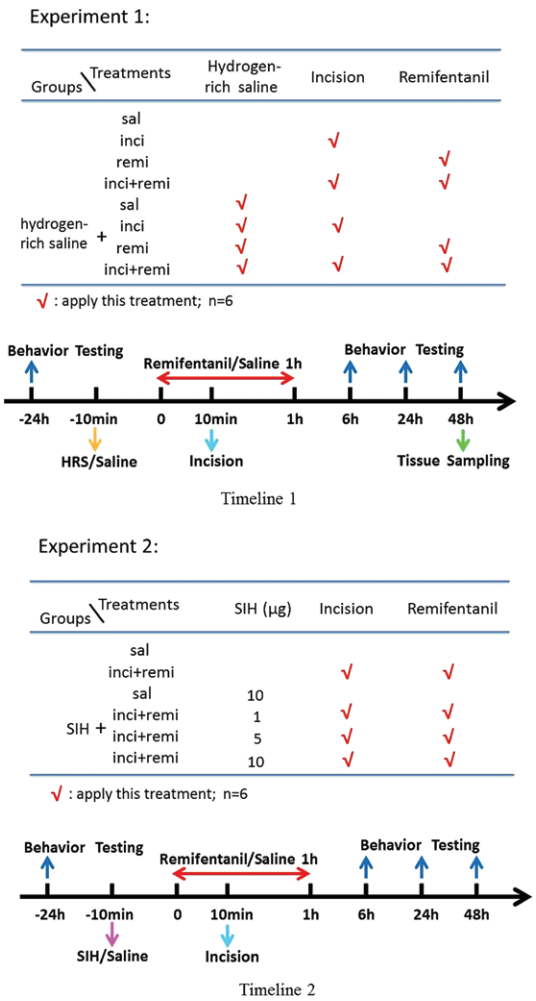

Fig. 1. Illustration of the experimental design. Experiment 1: To confirm whether peroxynitrite, divalent metal transporter 1, iron homeostasis are involved in remifentanil-induced postoperative hyperalgesia and whether removing peroxynitrite with hydrogen-rich saline prevents hyperalgesia. Animals were randomly divided into eight groups (n = 6). Behavior testing (mechanical and thermal hyperalgesia) was conducted at –24, 6, 24, 48 h after intravenous infusion in each group. Thermal hyperalgesia was measured 10 min after the determination of mechanical hyperalgesia in the same animals. Remifentanil (or saline) was intravenously infused at a rate of 1.0 μg · kg^{-1} · min^{-1} for 1 h. Incision was made 10 min after remifentanil infusion. Hydrogen-rich saline (10 ml/kg) was intraperitoneally injected 10 min before remifentanil infusion. Experiment 2: To confirm whether iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) attenuates hyperalgesia. Animals were randomly divided into six groups (n = 6). Behavior testing, remifentanil infusion, and incision were applied the same as experiment 1. SIH (1 μg, 5 μg, 10 μg) was intrathecally injected 10 min before remifentanil infusion. HRS = hydrogen-rich saline; inci = incision; inci + remi = incision + remifentanil; remi = remifentanil; sal = saline.

times with an interval of 10 min; the mean PWL was assessed from the average value of the three trials. A cutoff time of 25 s was adopted to prevent tissue damage.

PWL was measured 10 min after the determination of PWT in the same animals. PWL and PWT were measured at 24 h before surgery to obtain baseline values and subsequently at postoperative 6, 24, and 48 h. Thermal and mechanical hyperalgesia were defined as a significant ($P < 0.05$) reduction in PWL and PWT, respectively, compared with the baseline. After the last behavioral test, spinal cord tissues from the lower lumbar enlargement (L4–L6) were removed and processed for molecular biological analysis.

Immunoprecipitation and Western Blot Analysis of Protein

Protein extract from the L4–L6 segments of spinal cord was performed, as previously described.[13] Protein concentration was determined by bicinchoninic acid assay. The levels of 3-nitrotyrosine and nitrated MnSOD were assessed by immunoprecipitation and Western blot; DMT1(-)IRE and DMT1(+)IRE were measured by Western blot analysis. The two methods were performed as previously described.[38,39] In brief, protein samples and a protein molecular weight marker, PageRuler Prestained Standard (10–170 kDa; Millipore, Billerica, MA), were resolved in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. After blocking with 5% nonfat milk in phosphate-buffered saline and 0.05% Tween-20 for 2 h at room temperature, the membranes were incubated with rabbit anti-3-nitrotyrosine (1:500; Millipore), rabbit polyclonal anti-MnSOD (1:1000; Millipore), rabbit polyclonal anti-DMT1(-)IRE (1:500; Alpha Diagnostic International, San Antonio, TX), rabbit polyclonal anti-DMT1(+)IRE (1:500; Alpha Diagnostic International), and rabbit anti-β-actin (1:2,000; Cell Signaling Technology, Boston, MA) at 4°C overnight. The bands were incubated for 2 h with peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:2,000; Jackson ImmunoResearch, West Grove, PA). The immunoblots were visualized by enhanced chemiluminescence detection using a Bio-Rad GS-700 imaging system with software (Bio-Rad, Hercules, CA) and normalized to β-actin bands.

Measurement of Mitochondrial MnSOD Activity

The L4–L6 spinal cord tissues were homogenized with 10 mM phosphate-buffered saline (potential of hydrogen 7.4), sonicated on ice for 1 min (20 s, three times), and centrifuged at 1,200*g* for 10 min. The superoxide dismutase activity was determined by the nitroblue tetrazolium assay, as described previously.[39] The copper/zinc superoxide dismutase (CuZnSOD) activity was blocked in this assay by adding 2 mM sodium cyanide after preincubation for 30 min. The rate of nitroblue tetrazolium reduction was measured by spectrophotometer (DU 640B; Beckman Coulter, Brea, CA) at 560 nm. The quantity of protein required to suppress the rate of nitroblue tetrazolium reduction by 50% was defined as 1 U of enzyme activity. Enzymatic activity was expressed in units per milligram of protein.[39]

Immunohistochemistry

The L4–L6 spinal cords were removed, fixed in 4% paraformaldehyde in phosphate-buffered saline (potential of hydrogen 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 phosphate-buffered saline containing 5% normal goat serum for 30 min at room temperature to block nonspecific reactions. Sections were incubated overnight at 4°C with the primary antibody of rabbit polyclonal anti-DMT1(-)IRE (1:100; Alpha Diagnostic International), followed by biotinylated secondary antibody (1:300; Boster Biological Technology, Co., 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. For control experiment, immunostaining included a negative control (the primary antibody of DMT1(-)IRE was omitted, and an equal concentration of rabbit immunoglobulin G was substituted) and a positive control (1 μg/μl DMT1(-)IRE peptide applied directly to sections 5 μl; Alpha Diagnostic International). Preabsorption of the DMT1(-)IRE antibody (1 μg/μl, 1 μl) with DMT1(-)IRE peptide (1 μg/μl, 5 μl) showed no immunoreactivity in tissue sections.

Iron Histochemistry

Iron in spinal cord was detected by the Perl stain method according to the Prussian blue reaction.[40] Sections were incubated in 4% potassium ferrocyanide and 4% hydrochloric acid in double distilled water for 1 h at room temperature. After rinsing with double distilled water, sections were counterstained with nuclear fast red (Sun Biological Technology, Shanghai, China) for 2 min. Insoluble blue compounds were produced by the hydrochloric acid–catalyzed reaction of potassium ferrocyanide and ferric ions released from binding proteins.[40]

Atomic Iron Assay

The iron content of lumbar spinal cord was monitored by flame atomic absorption spectrophotometer (AA-6800; Shimadzu, Kyoto, Japan) at 248 nm, following digestion of tested tissues. To achieve dry mass, samples (0.1 to 0.2 g) were dried at 60°C for 12 h. Samples were first digested with 1 ml nitric acid (60%) at 100°C in water bath for 2 h, carrying on the digestion after the addition of 0.5 ml hydrogen peroxide (30%) for 0.5 h at boiling.[41] The completely dissolved residues were diluted to 10 ml with double distilled water before analysis. Atomic iron levels were calculated as micrograms per gram wet weight of tissue by comparing the absorbance to a range of standard concentrations of $FeSO_4$.

Statistical Analysis

On the basis of the results of a pilot study (unpublished data, January 2013, Rui-Chen Shu, Ph.D., Tianjin, China, continuous variable), we performed a power analysis to further examine the sample size. The mean PWL at baseline (–24 h) was 16.0 s, whereas under test conditions at 48 h, it was 16.2 s for control (sal group) and 11.2, 11.3, and 6.0 s for treatment groups (inci, remi, and inci + remi groups). Thus, we estimated a difference of at least 30% (error standard deviation = 1.3) between the control and the treatment groups. An *a priori* algorithm was used to compute the required sample size for performing repeated measures ANOVA. To find a statistically significant (α = 5%) difference with a statistical power of 0.8 between control and test groups at –24 and 48 h, a minimum of n = 4 animals per group was required. On the basis of this calculation, and to ensure accurate data, we have chosen to increase the sample size to 6. Animals were randomized to experimental conditions, and no data were lost to observation.

Using the Shapiro–Wilk test of normality, data were normal distribution and parametric statistics were applied. Homogeneity of variance was proved by the Levene test. In experiment 1, data from PWL, PWT, Western blot analysis, and spectrophotometer measurements were analyzed by one-way ANOVA with Dunnett *post hoc* comparisons. In experiment 2, time course data from behavior testing were analyzed by two-way repeated measures ANOVA with Bonferroni *post hoc* comparisons. Data were expressed as mean ± SD. Significant differences were defined as $P < 0.05$. All statistical analysis was performed using GraphPad Prism (release 5.04; GraphPad Software, La Jolla, CA).

Results

Peroxynitrite Formation in Spinal Cord Is Associated with Remifentanil-induced Postoperative Hyperalgesia

Before the surgery (–24 h), baseline numbers of nociceptive thresholds (PWL and PWT) were similar in all groups of rat ($P > 0.05$, n = 6 per group, fig. 2, A and B). At 48 h after surgery, incision (n = 6) and remifentanil (n = 6), respectively, resulted in the development of thermal ($P < 0.001$, fig. 2C) and mechanical ($P < 0.001$, fig. 2D) hyperalgesia when compared with saline rats (vehicle, n = 6). Moreover, remifentanil apparently facilitated hyperalgesia induced by incision (inci-remi *vs.* inci, 6.02 ± 0.72 s *vs.* 11.14 ± 0.92 s, 5.28 ± 0.78 g *vs.* 12.87 ± 1.29 g, respectively, $P < 0.001$ for each, n = 6, fig. 2, C and D). Three-nitrotyrosine represents a specific peroxynitrite-mediated protein modification, and therefore, the detection of 3-nitrotyrosine in proteins is widely used as a biomarker for endogenous formation of peroxynitrite.[42] When compared with vehicle (0.25 ± 0.05, n = 4 per group), the formation of 3-nitrotyrosine in spinal cord dramatically increased in rats receiving incision (0.51 ± 0.08), remifentanil (0.52 ± 0.06), especially in rats of remifentanil-induced postoperative hyperalgesia (1.22 ± 0.18, n = 4, $P < 0.01$, fig. 3A).

Removing Peroxynitrite by Intraperitoneal Delivery of Hydrogen-rich Saline Protects against Remifentanil-induced Postoperative Hyperalgesia

Hydrogen-rich saline was applied as a peroxynitrite decomposition catalyst in this study. Confirming previous reports,[20,23]

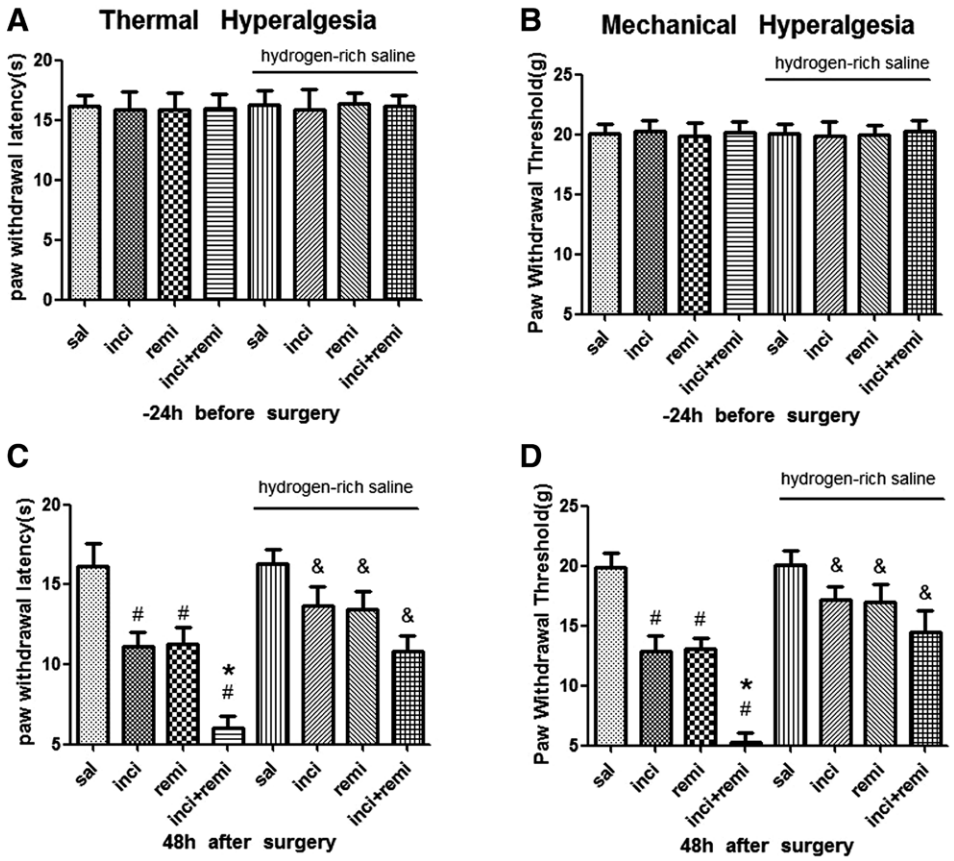

Fig. 2. Intraperitoneal delivery of hydrogen-rich saline prevents remifentanil-induced postoperative hyperalgesia. The baseline numbers of paw withdrawal latency (PWL) (*A*) and paw withdrawal threshold (PWT) (*B*) were similar in all groups. When compared with rats receiving saline (sal), incision (inci) and remifentanil (remi) significantly increased PWL (*C*) and PWT (*D*), respectively, at 48 h after surgery. Moreover, incision–remifentanil (inci + remi) treatment significantly enhanced thermal and mechanical hyperalgesia induced by incision. Intraperitoneal delivery of hydrogen-rich saline (10 ml/kg) attenuated remifentanil- and incision-depended hyperalgesia. Hydrogen-rich saline in rats receiving saline had no effect on PWL and PWT. Results are expressed as mean ± SD for n = 6 rats and analyzed by the one-way ANOVA with Dunnett *post hoc* comparisons. #$P < 0.01$ *vs.* saline; *$P < 0.01$ *vs.* incision; &$P < 0.01$ *vs.* corresponding non–hydrogen-rich saline group.

our data revealed that intraperitoneal delivery of hydrogen-rich saline (10 ml/kg, n = 4 per group) significantly reduced 3-nitrotyrosine formation when compared with corresponding groups untreated with hydrogen-rich saline ($P < 0.01$, n = 4, fig. 3A); it had no effect on rats receiving subcutaneous saline ($P > 0.05$, n = 4, fig. 3A). In addition, intraperitoneal delivery of hydrogen-rich saline (10 ml/kg, n = 6 per group) at 10 min before surgery protected against remifentanil-induced postoperative hyperalgesia ($P < 0.001$, n = 6, fig. 2, C and D). When given alone, hydrogen-rich saline did not affect baseline withdrawal thresholds ($P > 0.05$, n = 6, fig. 2, C and D). It demonstrated that peroxynitrite formation plays an important role in this setting.

Spinal Peroxynitrite Is Necessary for MnSOD Nitration and Inactivation

Peroxynitrite nitrates and inactivates mitochondrial MnSOD, the key enzyme eliminating superoxide, and thus, peroxynitrite is maintained at elevated level.[43] When compared with vehicle (n = 4), remifentanil-induced postoperative hyperalgesia was correlated with increased nitration (1.01 ± 0.1 *vs.* 0.19 ± 0.03, $P < 0.001$, fig. 3B) and decreased activity (50.3 ± 6.13 mU/μg *vs.* 208 ± 9.31 mU/μg, $P < 0.001$, fig. 3C) of mitochondrial MnSOD. Intraperitoneal hydrogen-rich saline (10 ml/kg, n = 4) attenuated MnSOD nitration and inactivation ($P < 0.001$, fig. 3, B and C). The activity of CuZnSOD were not affected in all groups ($P > 0.05$, n = 4 per group, fig. 3D).

DMT1(-)IRE-mediated Iron Accumulation Presents in Remifentanil-induced Postoperative Hyperalgesia

When compared with rats receiving saline (0.33 ± 0.06, n = 4 per group), incision (0.63 ± 0.13) and remifentanil (0.66 ± 0.09) led to elevated expression of DMT1(-)IRE in proteins of spinal cord individually ($P < 0.01$, n = 4, fig. 4A). Intraoperative infusion of remifentanil induced significant increase in DMT1(-)IRE expression in rats with incision (inci-remi *vs.* inci, 1.42 ± 0.19 *vs.* 0.63 ± 0.13, $P < 0.001$, n = 4, fig. 4A). However, spinal DMT1(+)IRE expression was unchanged by any treatment ($P > 0.05$, fig. 4B). In-line

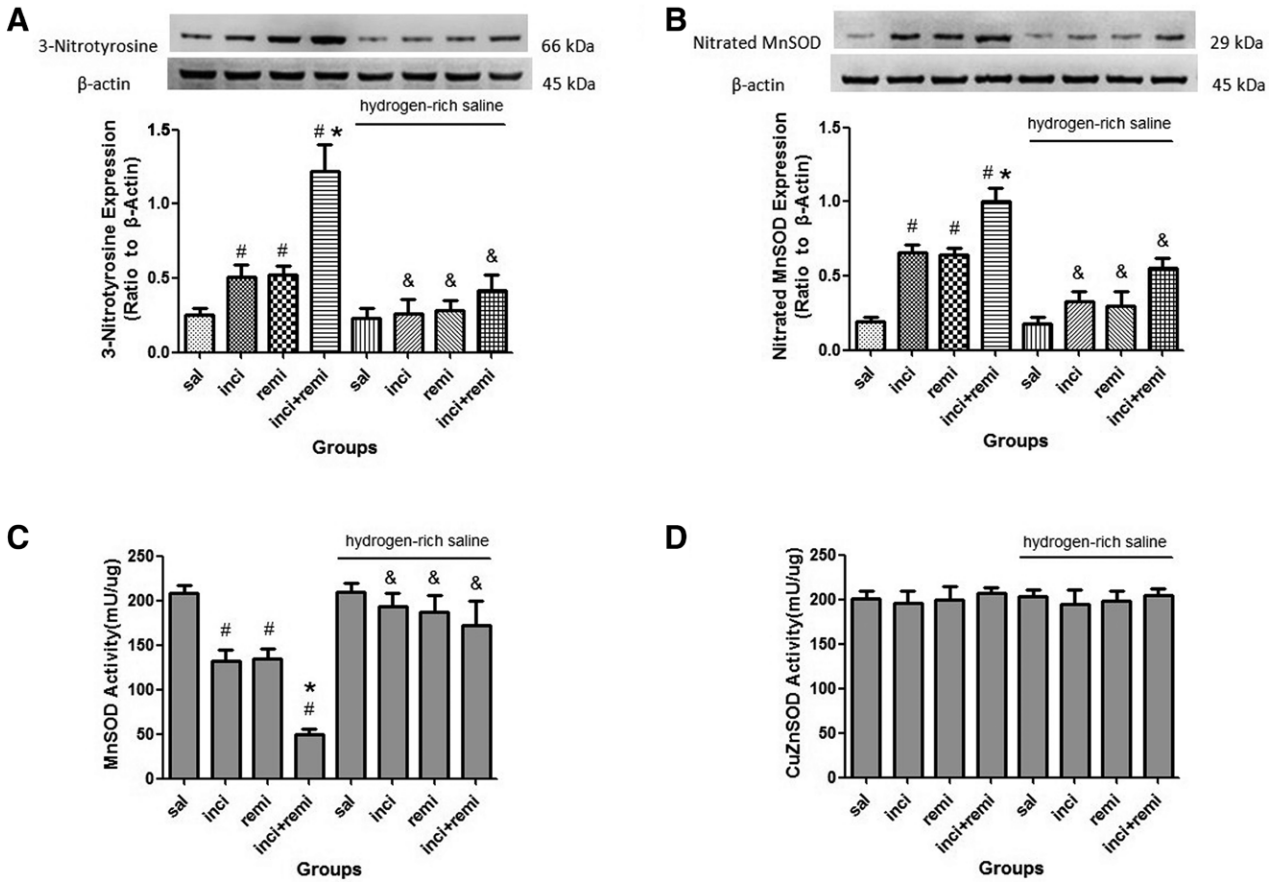

Fig. 3. Increased peroxynitrite formation and manganese superoxide dismutase (MnSOD) nitration and inactivation in remifentanil-induced postoperative hyperalgesia; prevention with hydrogen-rich saline. Three-nitrotyrosine is a biomarker of peroxynitrite. When compared with vehicle (sal), incision (inci) and remifentanil (remi) caused significant increase in 3-nitrotyrosine (*A*), nitrated MnSOD (*B*), and decrease in MnSOD activity (*C*). Incision–remifentanil group (inci + remi) had greater levels of 3-nitrotyrosine and nitrated MnSOD and less level of MnSOD activity than incision group. Intraperitoneal hydrogen-rich saline (10 ml/kg) prevented 3-nitrotyrosine formation (*A*), MnSOD nitration (*B*), and inactivation (*C*); it had no effect on rats receiving saline. Spinal copper/zinc SOD (CuZnSOD) activity was unchanged by any treatment (*D*). Results are expressed as mean ± SD for n = 4 rats. Data are analyzed using the one-way ANOVA with Dunnett *post hoc* comparisons. #$P < 0.01$ *vs.* saline; *$P < 0.01$ *vs.* incision; &$P < 0.01$ *vs.* corresponding non–hydrogen-rich saline group.

with above result, the presence of DMT1(-)IRE in spinal cord sections had similar changes (fig. 5). Immunostaining specificity controls were as shown in fig. 5, I, J, and K. There were greater levels of DMT1(-)IRE in rats receiving incision (n = 4 per group, fig. 5B), remifentanil (fig. 5C) than vehicle (fig. 5A), and it expressed highest in rats of remifentanil-induced postoperative hyperalgesia (fig. 5D).

Because DMT1(-)IRE is a transmembrane iron import channel,[24] our experiment showed that DMT1(-)IRE overexpression is associated with increased iron uptake in spinal cord. Iron concentration was quantified with the atomic iron analysis. When compared with vehicle (sal, 5.26 ± 0.61 μg/g, n = 6 per group, table 1), spinal iron concentration significantly elevated in incision (inci, 8.73 ± 0.92 μg/g, $P < 0.01$, table 1) and remifentanil (remi, 9.15 ± 0.58 μg/g, $P < 0.01$, table 1) groups individually. Intraoperative infusion of remifentanil dramatically increased iron content in rats that underwent incision (inci-remi *vs.* inci, 12.87 ± 1.14 μg/g *vs.* 8.73 ± 0.92 μg/g, $P < 0.01$, table 1). Iron histochemistry was used to detect iron distribution in dorsal horn of spinal cord (fig. 6). Consistent with results of atomic iron analysis, iron stain was inconspicuous in vehicle (n = 4, fig. 6A), while there were iron accumulation in groups of incision (n = 4, fig. 6B) and remifentanil (n = 4, fig. 6C), especially in incision–remifentanil group (n = 4, fig. 6D).

DMT1(-)IRE-mediated Iron Accumulation Is Regulated by Peroxynitrite in Spinal Cord

Intraperitoneal delivery of hydrogen-rich saline (10 ml/kg, n = 4 per group) significantly reduced DMT1(-)IRE expression in proteins ($P < 0.001$, fig. 4A) and in spinal cord sections (fig. 5, F–H) when compared with corresponding non–hydrogen-rich saline-treated groups (n = 4, figs. 4A and 5, B–D) but did not affect DMT1(-)IRE expression on rats receiving saline (n = 4, figs. 4A and 5E). Meanwhile, intraperitoneal hydrogen-rich saline (10 ml/kg, n = 6 per group)

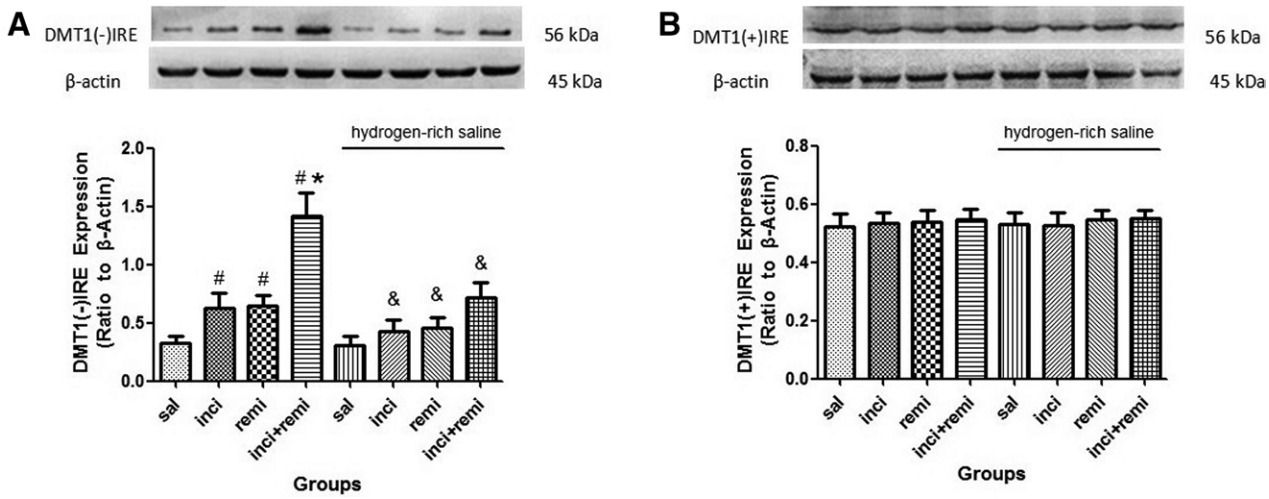

Fig. 4. Divalent metal transporter 1 without iron-responsive element [DMT1(-)IRE], but not DMT1 with IRE [DMT1(+)IRE], is associated with remifentanil-induced postoperative hyperalgesia. When compared with vehicle (sal), incision (inci) and remifentanil (remi) caused a significant increase of DMT1(-)IRE expression (*A*) in proteins of spinal cord. Incision–remifentanil (inci + remi) had higher level of DMT1(-)IRE than incision. Intraperitoneal hydrogen-rich saline (10 ml/kg) reduced DMT1(-)IRE expression (*A*) but not its vehicle. DMT1(+)IRE expression (*B*) was unaffected by incision and remifentanil treatments. Results are expressed as mean ± SD for n = 4 rats. Data are analyzed using the one-way ANOVA with Dunnett *post hoc* comparisons. #$P < 0.01$ *vs.* saline; *$P < 0.01$ *vs.* incision; &$P < 0.01$ *vs.* corresponding non–hydrogen-rich saline group.

suppressed iron accumulation in remifentanil-induced postoperative hyperalgesia (H + inci + remi *vs.* inci + remi, 8.24 ± 1.06 μg/g *vs.* 12.87 ± 1.14 μg/g, $P < 0.01$, n = 6, table 1); it had no effect on saline vehicle (5.08 ± 0.82 μg/g, n = 6, $P > 0.05$, table 1). This result was also supported by spinal iron stain (n = 4, fig. 6, E–H).

Iron Chelation Prevents Remifentanil-induced Postoperative Hyperalgesia

To verify the contribution of iron accumulation to remifentanil-induced postoperative hyperalgesia, we used intrathecal delivery of a high-affinity iron chelator, SIH.[29,34] When compared with corresponding non–SIH-treated groups (n = 6), intrathecal administration of SIH (1–10 μg, n = 6) prevented remifentanil-induced postoperative thermal ($P < 0.01$, fig. 7A) and mechanical hyperalgesia ($P < 0.01$, fig. 7B) in a dose-dependent manner. SIH had no effect on rats receiving subcutaneous saline ($P > 0.05$, n = 6, fig. 7, A and B).

Discussion

The dosage of remifentanil (1.0 μg · kg^{-1} · min^{-1}, 60 min) used in this experiment was based on our previous study.[36] According to equivalent dose conversion table between the species,[44] the dose of rat is 6.25 times of human to achieve the same pharmacodynamic effect. Therefore, the dose of 1.0 μg · kg^{-1} · min^{-1} in rat converting to human dose is 0.16 μg · kg^{-1} · min^{-1}, which is within the clinically accepted doses. With a common used rat model of remifentanil-induced postoperative hyperalgesia,[45] our results suggest that excess formation of peroxynitrite is of great significance in hyperalgesia (fig. 8). Inhibition of peroxynitrite production using the well-characterized antioxidant, hydrogen-rich saline, protects against the development of thermal and mechanical hypersensitivity. Nitrated and inactivated enzymatic source of MnSOD after the peroxynitrite formation results in mitochondrial dysfunction. However, the cytosolic CuZnSOD activity is unaffected by any treatment. In addition, our data reveal that, for the first time, aberrant peroxynitrite production facilitates iron accumulation *via* activating DMT1(-)IRE, which contributes to remifentanil-induced postoperative hyperalgesia *in vivo*, while DMT1(+)IRE is not involved in this setting. Remarkably, selective iron chelator SIH attenuates remifentanil-induced postoperative hyperalgesia in a dose-dependent manner. These findings provide possibilities for the prevention of hyperalgesia and substantial benefit to patients undergoing opioid therapy.

Peroxynitrite is generated by the reaction of superoxide and nitric oxide with a half-life of about 10 ms and can easily diffuse through membranes.[43] Three-nitrotyrosine represents a specific peroxynitrite-mediated protein modification *in vivo*, and therefore, 3-nitrotyrosine is widely used as a biomarker for endogenous formation of peroxynitrite.[42] Previous studies have demonstrated that peroxynitrite is a critical determinant of pain states, including chemotherapy-induced peripheral neuropathy,[12,14] inflammatory pain hypersensitivity,[13] and morphine antinociceptive tolerance.[17,38] Mitochondria are the primary site for energy generation, as well as reactive oxygen/nitrogen species production. Peroxynitrite-dependent nitration and inactivation of MnSOD lead to mitochondrial dysfunction, which underlies cold and mechanical hyperalgesia,[15,46,47] and trigger a feedforward mechanism to maintain elevated peroxynitrite, as well as nitroxidative stress. Peroxynitrite also nitrates mitochondrial

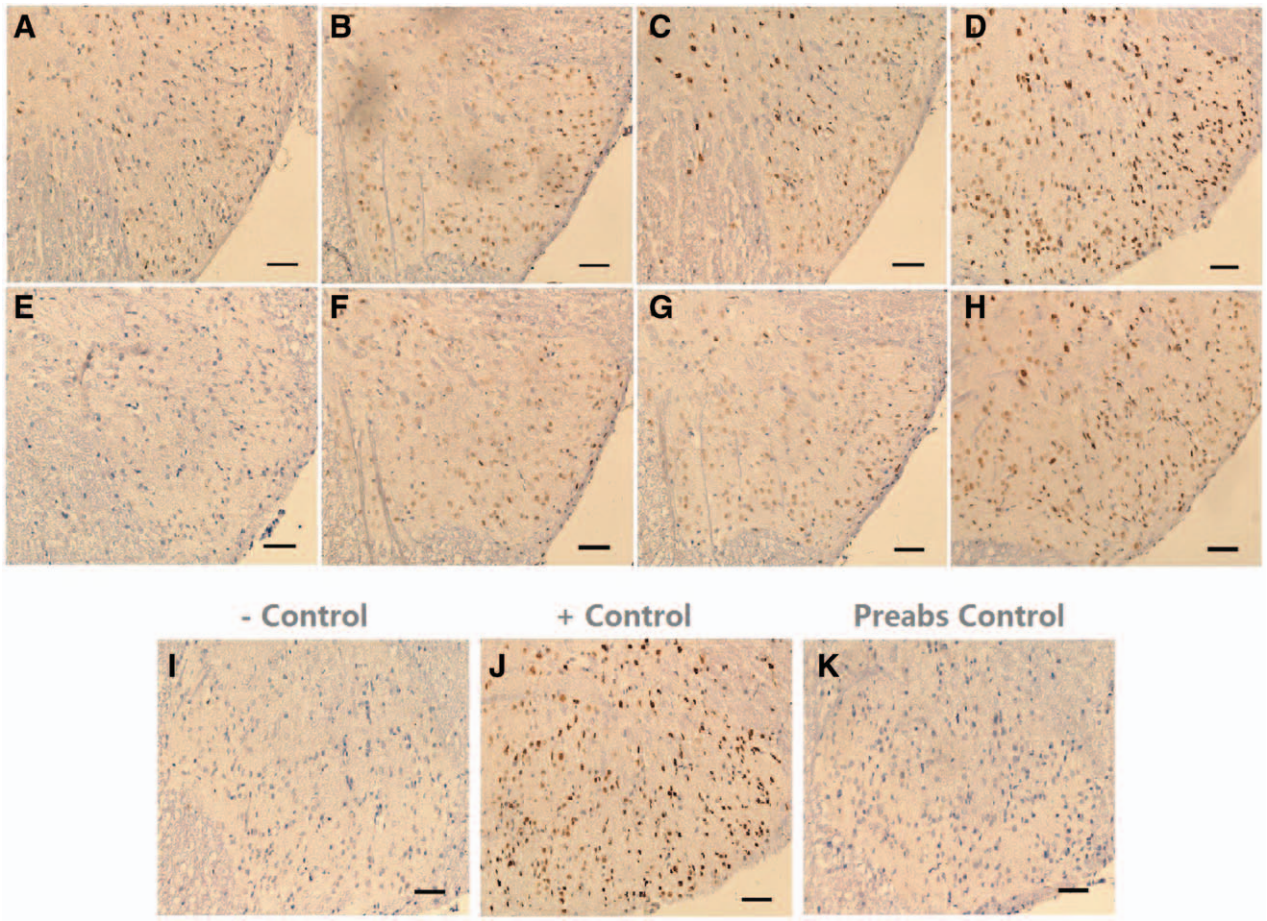

Fig. 5. The spinal peroxynitrite pathway is required for the activation of divalent metal transporter 1 without iron-responsive element [DMT1(-)IRE]. Representative immunohistochemistry micrographs of dorsal horn of L4–L6 spinal cord showed that DMT1(-)IRE presents the brown staining and mainly locates at the nuclear of neurons. When compared with vehicle (*A*), the expression of DMT1(-)IRE slightly increased in rats receiving incision (*B*) and remifentanil (*C*) individually and dramatically increased in incision–remifentanil rats (*D*). Intraperitoneal delivery of hydrogen-rich saline (10 ml/kg) blocked the increasing DMT1(-)IRE expression respectively (*F*, *G*, *H*) but not its vehicle (*E*). (*I–K*), The DMT1(-)IRE negative control (– Control) (*I*) and preabsorption control (Preabs Control) (*K*) showed absence of labeling, while the DMT1(-)IRE positive control (+ Control) (*J*) provided intense immunoreactivity. n = 4 rats per group, *scale bar* = 50 μm.

Table 1. Fe Concentration in Spinal Cord (Micrograms per Gram Wet Tissue)

Treatment	Fe Concentration (μg/g)
Sal	5.26 ± 0.61
Inci	8.73 ± 0.92*
Remi	9.15 ± 0.58*
Inci + remi	12.87 ± 1.14*†
H + sal	5.08 ± 0.82
H + inci	6.59 ± 0.85‡
H + remi	6.93 ± 0.57‡
H + inci + remi	8.24 ± 1.06‡

Mean ± SD for n = 6 rats. Incision (inci) and remifentanil (remi), especially their combination (inci-remi), caused an increase of iron level in spinal cord compared with vehicle (sal). Hydrogen-rich saline (H, 10 ml/kg) lighten the increased iron level, respectively, but not its vehicle. Data were analyzed by one-way ANOVA with Dunnett *post hoc* comparisons.

*P < 0.01 *vs.* vehicle. †P < 0.01 *vs.* incision. ‡P < 0.01 *vs.* corresponding non–hydrogen-rich saline groups.

iron-sulfur enzymes by destabilizing iron-sulfur cluster, which results in mitochondrial dysfunction and the release of iron, leading to iron accumulation.[48,49] The CuZnSOD activity is not changed, which is coincident with the fact that peroxynitrite has no effect on the catalytic activity of CuZnSOD.[50] Furthermore, peroxynitrite promotes neuroinflammation by activating nuclear factor kappa B (NF-κB), extracellular signal–regulated kinase, and P38 mitogen–activated protein kinases pathways and modulating pro- and anti-inflammatory cytokines in spinal cord.[12,17] More importantly, peroxynitrite enhances glutamatergic signaling through nitration and inactivation key proteins, in particular glutamate transporter 1 and glutamine synthetase, leading to increased glutamate and rapid alterations in synaptic transmission.[12,16] Neuroinflammation and enhanced glutamatergic signaling have been demonstrated to be critical in the development of opioid-induced hyperalgesia.[30]

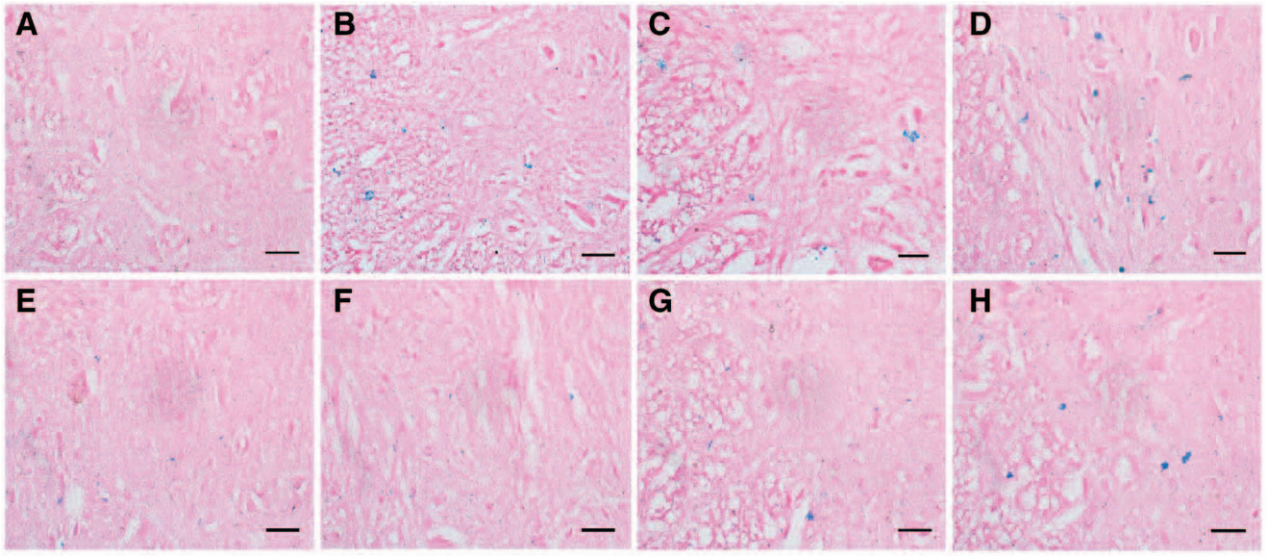

Fig. 6. Peroxynitrite-activated divalent metal transporter 1 without iron-responsive element [DMT1(-)IRE] overexpression leads to abnormal iron accumulation in remifentanil-induced postoperative hyperalgesia. Iron accumulation in spinal cord was shown as blue deposits in Perl stain micrographs. Hardly any iron accumulation was seen in the vehicle (*A*), there were mild iron accumulation in rats receiving incision (*B*) and remifentanil (*C*) individually, and pronounced iron accumulation in incision–remifentanil-treated rats (*D*). Intraperitoneal delivery of hydrogen-rich saline (10 ml/kg) protected against abnormal iron accumulation, respectively (*F*, *G*, *H*), but not its vehicle (*E*). n = 4 rats per group, *scale bar* = 50 μm.

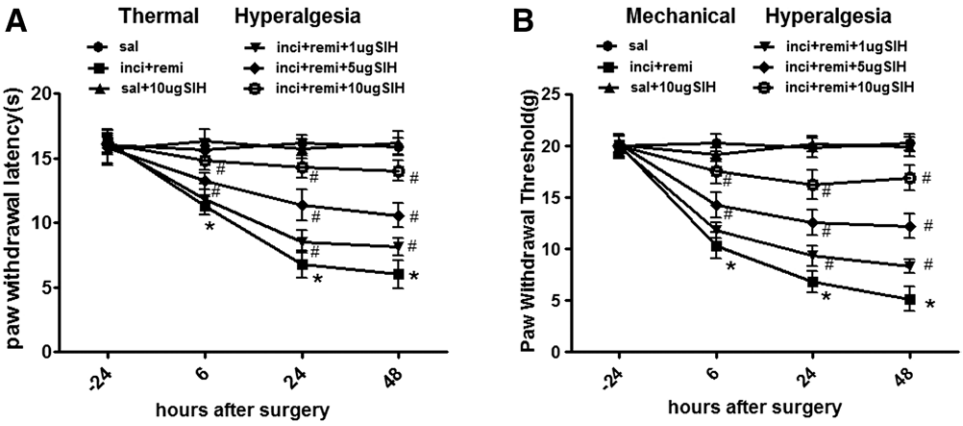

Fig. 7. Salicylaldehyde isonicotinoyl hydrazone (SIH) prevents remifentanil-induced postoperative hyperalgesia in a dose-dependent manner. When compared with vehicle (●), remifentanil (■) resulted in a time-dependent development of postoperative thermal (*A*) and mechanical (*B*) hyperalgesia. Intrathecal delivery of SIH (1 μg, ▼; 5 μg, ◆; 10 μg, ○) significantly attenuated the development of hyperalgesia in a dose-dependent manner (*A*, *B*). SIH in rats receiving saline (▲) had no effect on nociceptive thresholds. Results are expressed as mean ± SD for n = 6 rats and analyzed by the two-way repeated measures ANOVA with Bonferroni comparisons. **P* < 0.01 for remifentanil *vs.* vehicle and #*P* < 0.01 for remifentanil *vs.* remifentanil + SIH. inci = incision; remi = remifentanil; sal = saline.

We recently found that increasing NMDA receptor subunits (NR1 and NR2B) trafficking from the intracellular pool to surface pool is responsible for remifentanil-induced postoperative hyperalgesia.[35] Overactivation of NMDA receptor causes neurotoxicity, which has been implicated in numerous pain states.[51] DMT1-mediated iron overload is suggested to participate in NMDA neurotoxicity, and the toxic effect can be blocked by selective iron chelation.[29] Accordingly, we hypothesized that DMT1-mediated iron accumulation may be also associated with pathophysiological pain, including remifentanil-induced postoperative hyperalgesia (fig. 8).

Consistent with the aberrant iron metabolism hypothesis, our results clearly demonstrate that peak remifentanil-induced hyperalgesia is correlated with iron accumulation regulated by DMT1(-)IRE but not DMT1(+)IRE. Of note, the removal of iron with SIH protects against thermal and mechanical hypersensitivity. To confirm whether there is connection between peroxynitrite and iron accumulation in hyperalgesia, we use hydrogen-rich saline that imparts selectivity for peroxynitrite decomposition without adverse effects.[18] Interestingly, intraperitoneal delivery of hydrogen-rich saline obviously suppresses the overexpression of

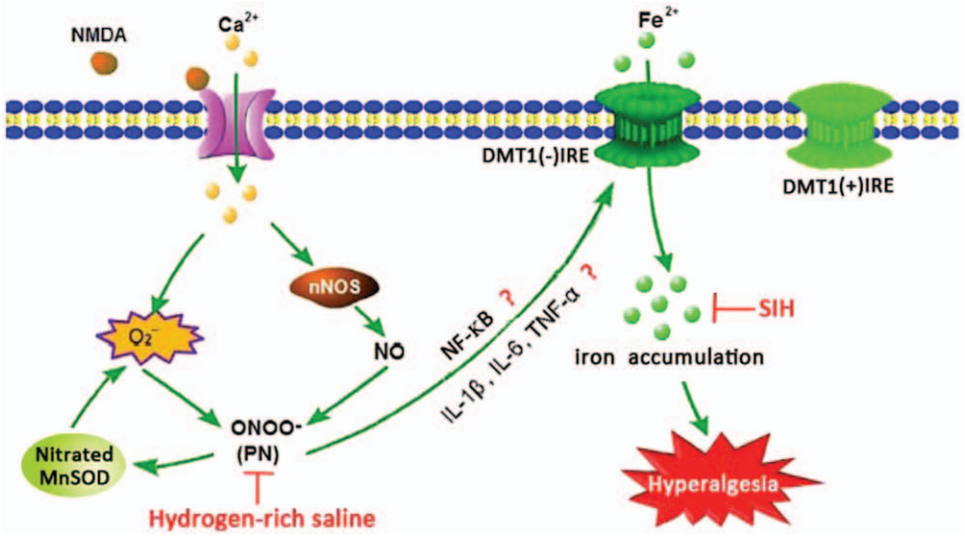

Fig. 8. Schematic illustration of proposed PN-DMT1(-)IRE-iron accumulation signaling in remifentanil-induced postoperative hyperalgesia. Activated glutamate-NMDA receptor (trafficking from the intracellular pool to surface pool) causes cellular calcium influx, *via* promoting the interaction between superoxide and nitric oxide, leading to PN formation. PN results in MnSOD nitration, forming excessive superoxide and triggering a feedforward mechanism to maintain elevated PN. PN activates DMT1(-)IRE and induces abnormal iron accumulation, leading to the development of hyperalgesia. Thus, PN decomposition with hydrogen-rich saline and iron chelation inhibit hyperalgesia. Possible mechanisms by which PN facilitates DMT1(-)IRE include NF-κB activation and elevated levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α. Ca^{2+} = calcium ion; DMT1(+)IRE = divalent metal transporter 1 with iron-responsive element; DMT1(-)IRE = divalent metal transporter 1 without iron-responsive element; Fe^{2+} = ferrous ion; IL-1β = interleukin-1 beta; IL-6 = interleukin-6; MnSOD = manganese superoxide dismutase; NF-κB = nuclear factor kappa B; NMDA = *N*-methyl-D-aspartate; nNOS = neuronal nitric oxide synthase; NO = nitric oxide; O_2^- = superoxide; PN = peroxynitrite; SIH = salicylaldehyde isonicotinoyl hydrazone; TNF-α = tumor necrosis factor-alpha.

DMT1(-)IRE and iron overload. These findings reveal that DMT1(-)IRE-mediated iron accumulation is the downstream of peroxynitrite in the development of remifentanil-induced postoperative hyperalgesia.

The mechanisms by which peroxynitrite regulates DMT1 expression are unknown; however, we believe activated NF-κB pathway and enhanced proinflammatory cytokines that are prominent factors in opioid-induced hyperalgesia[30] are likely involved in this (fig. 8). Peroxynitrite activates NF-κB pathway by inhibition of mitochondrial respiration and redox reaction with related components. For example, systemic administration of peroxynitrite decomposition catalyst can block the phosphorylation and nuclear translocation of NF-κB p65 subunit.[17] Moreover, peroxynitrite directly activates NF-κB through nitration and degradation of IκB-α, the regulatory molecule responsible for secluding the activation of NF-κB, giving rise to the release of NF-κB.[17,52] Using the gene cluster analysis, DMT1 is suggested to be one of the three NF-κB target genes, which are associated with oxygen homeostasis and response to oxidative stress, as well as iron homeostasis and response to metal ions,[31] and DMT1 promoter contains NF-κB–binding sites.[53,54] The fact that NF-κB regulates iron transporter DMT1 expression is also verified in inflammatory and neuropathic disorders.[31,55] In addition, enhanced proinflammatory cytokines may be linked to the process of peroxynitrite regulating DMT1 in remifentanil-induced postoperative hyperalgesia. It is reported that peroxynitrite elevates the levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha in neuropathic pain.[12,17] These cytokines have been demonstrated to promote DMT1 expression and iron uptake in pancreatic β-cell[31] and central nervous system cells,[56] respectively.

DMT1 is highly expressed in mammalian neuronal cells and is composed of 12 membrane-spanning domains subject to alternative splicing.[24] The mRNA 5′ splicing produces 1A or 1B DMT1 isoforms, and the mRNA 3′ splicing generates (+)IRE or (-)IRE isoforms.[25] DMT1(+)IRE is post-transcriptionally regulated by the IRE/iron regulatory protein system, which adjusts its expression according to iron level, while DMT1(-)IRE is not susceptible to iron content.[57] Available studies reveal that DMT1(-)IRE, but not DMT1(+)IRE, is entirely responsible for the import of iron released from transferrin to the early endosomal lumen.[27] A broad up-regulation of DMT1(-)IRE, rather than DMT1(+)IRE, is associated with 1-methyl-4-phenylpyridinium–induced apoptosis in MES23.5 dopaminergic cell.[28] Notably, increasing DMT1(-)IRE also plays a critical role in L-3,4-dihydroxyphenylalanine neurotoxicity[26] and brain ischemia injury.[55] It is therefore possible that DMT1(-)IRE is more involved in mediating aberrant iron uptake and neuronal cell death. In-line with this hypothesis, our data reveal that enhanced DMT1(-)IRE expression in spinal cord is relevant to remifentanil-induced postoperative hyperalgesia. DMT1(+)IRE expression appears unchanged, possibly as a

consequence of its negative posttranscriptional regulation by iron increase.[25,57]

Iron is essential for life yet toxic if homeostasis is perturbed. Although iron deficiency can result in physical impairment, iron accumulation has also been implicated in a variety of well-known diseases, including heart failure, diabetes, cancer, and neurodegenerations.[31–34,58] Owing to its ability of donating electrons to oxygen, excessive iron causes oxidative stress, as well as subsequent DNA and mitochondrial damage, lipid peroxidation, and protein modification *via* the Fenton reaction,[59] which are well-characterized factors that contributed to opioid-induced hyperalgesia.[30] Moreover, iron overload hinders neurotransmitter synthesis and axons myelination.[59] Iron chelator treatment prevents remifentanil-induced postoperative hyperalgesia, manifesting the critical role of iron accumulation in the progress of this disease. Orally effective and well-tolerated iron chelators have been developed and now in routine clinical use for iron overload–related diseases.[60] With respect to therapeutic efficacy, iron chelator should undergo clinical testing for the prevention of opioid-induced hyperalgesia.

To confirm the role of peroxynitrite and downstream targets, we use hydrogen-rich saline, a free radical scavenge recently identified by good many studies. Ohsawa *et al.*[19] reported, for the first time, that hydrogen markedly reduced oxidative stress in the rat model of brain ischemia/reperfusion injury. Also, the antioxidative activity of hydrogen is verified in diverse diseases, such as diabetes, neurodegenerations, organ transplantation, and sepsis.[21,22,61,62] In agreement with previous studies, our results reveal that hydrogen-rich saline inhibits peroxynitrite formation and prevents remifentanil-induced postoperative hyperalgesia. Hydrogen acts as a therapeutic antioxidant by selectively eliminating peroxynitrite and hydroxyl radical rather than other reactive oxygen/nitrogen species, which possess physiological roles.[19,20,23] Attributing to its ability to rapidly diffuse across membranes, hydrogen can react with cytotoxic reactive oxygen species.[18] Previous studies demonstrated that hydrogen exerts well therapeutic effect at a low dosage and through multiple delivery methods, including gas inhalation, drinking hydrogen dissolved water, and hydrogen-rich saline injection without adverse effects.[18,20] Of note, the kinetics of the hydrogen has been detected in our laboratory study.[63] In rat model of remifentanil-induced hyperalgesia, hydrogen concentration in blood increased 5 min, peaked 15 min, and returned to basal level 45 min after hydrogen-rich saline administration. In addition, hydrogen-rich saline injection at less than 10 ml/kg has no significant effects on arterial blood gas. Importantly, the amount of hydrogen dissolved in arterial blood was more than that in vein, suggesting that hydrogen might be incorporated into "at-risk" tissues (spinal cord) to offer antinociception. Time course of hydrogen concentration in blood after treatment was also indicated by some other studies.[19,64] Minimizing interference in normal cellular functions meanwhile affording selectivity for peroxynitrite decomposition makes molecular hydrogen more attractive for studying the mechanism of peroxynitrite in remifentanil-induced postoperative hyperalgesia. More significantly, hydrogen treatment may be a beneficial approach for the clinical therapy of pain, including opioid-induced hyperalgesia.

In summary, aberrant peroxynitrite-dependent iron accumulation *via* DMT1(-)IRE contributes to remifentanil-induced postoperative hyperalgesia *in vivo*, hydrogen-rich saline, and iron chelation protect against the development of hyperalgesia. This concept may be generalizable to other pain states related to peroxynitrite overexpression and may open avenues for novel therapies of pain.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China, Beijing, China (nos. 81371245, 81071059, and 81100984), and Science and Technology Supported Key Project of Tianjin, Tianjin, China (12ZCZDSY03000).

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Guo-Lin Wang: Department of Anesthesiology, Tianjin Medical University General Hospital, 154 Anshan Road, Tianjin 300052, P. R. China. wang_guolin@hotmail.com. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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