



## Research report

## Involvement of TRPV4-NO-cGMP-PKG pathways in the development of thermal hyperalgesia following chronic compression of the dorsal root ganglion in rats

Xin-Li Ding<sup>a,1</sup>, Yong-Hui Wang<sup>a,1</sup>, Li-Ping Ning<sup>a</sup>, Yang Zhang<sup>a</sup>,  
Hong-You Ge<sup>b</sup>, Hong Jiang<sup>c</sup>, Rong Wang<sup>c</sup>, Shou-Wei Yue<sup>a,\*</sup><sup>a</sup> Department of Physical Medicine & Rehabilitation, Qilu Hospital, Medical School of Shandong University, Jinan 250012, China<sup>b</sup> Center for Sensory-Motor Interaction, Department of Health Science and Technology, Aalborg University, Aalborg, Denmark<sup>c</sup> Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Public Health, Qilu Hospital, Shandong University, Jinan 250012, China

## ARTICLE INFO

## Article history:

Received 25 July 2009

Received in revised form

18 November 2009

Accepted 20 November 2009

Available online 3 December 2009

## Keywords:

TRPV4

Nitric oxide

Cyclic guanosine monophosphate

Protein kinase G

Neuropathic pain

Thermal hyperalgesia

## ABSTRACT

The aim of the present study was to test the hypothesis that the TRPV4-NO-cGMP-PKG cascade is involved in the maintenance of thermal hyperalgesia following chronic compression of the dorsal root ganglion (DRG) (the procedure hereafter termed CCD) in rats. CCD rats showed thermal hyperalgesia and increased nitrite production. Intrathecal administration of ruthenium red (TRPV4 antagonist, 0.1–1 nmol), TRPV4 antisense ODN (TRPV4 AS, 40 μg, daily for 7 days), N<sup>G</sup>-L-nitro-arginine methyl ester (L-NAME, inhibitor of NO synthase, 30–300 nmol), 1H-[1,2,4]-oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, a soluble guanylate cyclase inhibitor, 50–100 nmol) or 8-(4-Chlorophenylthio) guanosine 3',5'-cyclic Monophosphothioate, Rp-Isomer sodium salt (Rp-8-pCPT-cGMPS, a PKG inhibitor, 25–50 nmol) induced a significant ( $P < 0.001$ ) and dose-dependent increase in the paw withdrawal latency (PWL) compared with control rats, respectively. Ruthenium red (1 nmol), TRPV4 AS (40 μg, daily for 7 days) or L-NAME (300 nmol) decreased nitrite (an index of nitric oxide formation) in the DRG of CCD rats. In addition, the phorbol ester 4α-phorbol 12,13-didecanoate (4α-PDD, TRPV4 synthetic activator, 1 nmol), co-administered with L-NAME (300 nmol), attenuated the suppressive effect of L-NAME on CCD-induced thermal hyperalgesia and nitrite production. Our data suggested that the TRPV4-NO-cGMP-PKG pathway could be involved in CCD-induced thermal hyperalgesia.

© 2009 Elsevier B.V. All rights reserved.

## 1. Introduction

Chronic compression of the dorsal root ganglion (DRG) (the procedure hereafter termed CCD) in animals, a typical model of neuropathic pain, mimics clinical disc herniation and spinal canal stenosis in humans. CCD rats show ipsilateral spontaneous pain, mechanical allodynia, and thermal hyperalgesia. In association with these behavioral effects, an increased excitability of neuronal somata in the compressed ganglion has been shown, as evidenced by spontaneous activity, lowered rheobase and action potential thresholds [18,36–39]. Cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) and cyclic guanosine monophosphate (cGMP)-dependent protein kinase G (PKG) pathways, both second messenger signals, have been shown to participate in maintaining neuronal hyperexcitability and behavioral hyperalgesia in CCD treatment [38]. Moreover, several types

of ion channels, such as voltage-gated Na<sup>+</sup> and K<sup>+</sup> [20,44], hyperpolarization-activated cation current [55], and TRPV4 [59] have been reported to mediate detection and transmission of nociceptive stimuli in CCD neurons. However, to date, the detailed mechanisms of nociceptive signal transfer and the correlation between the ion channels and the second messenger signals in behavioral hyperalgesia remain unclear in the CCD model of neuropathic pain in rats.

TRPV4 is a polymodal receptor that is activated by hypotonicity, mechanical stimuli, heat, phorbol esters, low pH, citrate, anandamide (AEA) and its LOX metabolite arachidonic acid (AA), and bisandrographolide A (BAA) [28,35,53]. TRPV4 is involved in mechanical hyperalgesia and allodynia, as revealed by enhanced responses to hypotonic or hypertonic test stimuli after sensitization by PGE<sub>2</sub> [1,2]. TRPV4 knockdown studies show abnormal osmotic regulation and increased nociceptive threshold to pressure with unchanged response to heat and touch [22,41]. TRPV4<sup>-/-</sup> knockout mice also show reduced thermal hyperalgesia in inflammatory pain [49]. Moreover, TRPV4 has been shown to contribute to mechanical and hypotonic hyperalgesia in neuropathic pain models, such as vincristine-induced neuropathy

\* Corresponding author. Tel.: +86 531 82169362; fax: +86 531 86927544.  
E-mail addresses: [drg98@hotmail.com](mailto:drg98@hotmail.com), [xinlidingaa@163.com](mailto:xinlidingaa@163.com) (S.-W. Yue).

<sup>1</sup> These authors contributed equally to this work.

(chemotherapy), paclitaxel-induced neuropathy (chemotherapy), streptozotocin-induced neuropathy (a model for diabetes), 2',3'-dideoxycytidine-induced neuropathy (AIDS therapy), and alcohol withdrawal-induced neuropathy (a model of binge drinking) [3,4]. While it is recognized that TRPV4 contributes to mechanical and hypotonic hyperalgesia in neuropathic and inflammatory pain [1–4], TRPV4 is also reported to be involved in thermal hyperalgesia in inflammatory pain [49]. However, whether TRPV4 participates in thermal hyperalgesia in neuropathic pain is unknown.

TRPV4, with its gene and protein expression in DRG increased after CCD, contributes to mechanical hyperalgesia and exhibits robust  $\text{Ca}^{2+}$  entry on exposure to a hypotonic milieu or a phorbol ester, 4 $\alpha$ -PDD [21,59]. Nitric oxide synthase (NOS) is  $\text{Ca}^{2+}$ -dependent and is responsible for the synthesis of NO from L-arginine [16,29]. NO has been shown to be a key mediator of nociceptive activity in animal pain models [23,34,46,47]. TRPV4 synthetic activator, 4 $\alpha$ -PDD, and hypoosmotic stimulation could induce NO production in outer hair cells of the guinea pig cochlea, while NO production is inhibited by ruthenium red, an inhibitor of TRPV4 [43]. TRPV4, as an osmosensory transducer, may modulate downstream effectors via glutamatergic synapses [7]. NO is also reported to increase glutamate release through cGMP-PKG signaling pathways [19,31]. Further, 4 $\alpha$ -PDD and hypoosmotic stimulation induce TRPV4-dependent CGRP release [17] and an increase in NO is associated with up-regulation of CGRP synthesis and release [6,9,12,15,40]. TRPV4 may thus be associated with NO-cGMP-PKG pathways. Moreover, NO-cGMP [32], cGMP-protein kinase G (PKG) [38], and NO-cGMP-PKG [45,48] pathways have been found to contribute to hyperalgesia in neuropathic and inflammatory pain. We therefore hypothesized that the activation of TRPV4 in DRG initiates an influx of  $\text{Ca}^{2+}$ , stimulating  $\text{Ca}^{2+}$ -dependent NOS, thereby activating the NO-cGMP-PKG pathway, which in turn enhances the release of glutamate and CGRP. This pathway may contribute to nociceptive response following CCD. In the present study, we evaluated the role of TRPV4-NO-cGMP-PKG pathways in thermal hyperalgesia in the CCD model by examining the effects of TRPV4 knockdown on behavioral responses and on NO production in the DRG.

## 2. Materials and methods

### 2.1. Animal and surgical procedure

The study complied with the Chinese Institutional Care Committee for the use of animals and was performed in accordance with the Helsinki declaration. All efforts were made to minimize suffering and the number of animals used. Adult male Wistar rats weighing 150–180 g (Shandong University Lab Animal Center, Jinan, China) were housed in a controlled environment (12 h light/dark cycle, room temperature  $23 \pm 2^\circ\text{C}$ , 50–60% relative humidity), with free access to food and water for at least 7 days before surgery. All experiments took place during the light period between 8:00 and 17:00 h in a quiet room. Rats were randomly divided into CCD and sham groups. In the CCD rats, under pentobarbital sodium anesthesia (Nembutal, 50 mg/kg ip), the transverse process and intervertebral foramina of L4 and L5 were exposed unilaterally as previously described [18,36]. A stainless steel L-shaped rod (0.63-mm diameter and 4-mm length) was inserted into each foramen, one at L4 and the other at the L5 level, to compress the DRG. The muscle and skin layers were then sutured. Penicillin was injected to prevent infection. The sham group underwent the same surgical procedure as described, but without the insertion of the rods. The animals did not show any autotomy, nor was there a complete loss of sensation following the surgery.

### 2.2. Antisense oligodeoxynucleotide treatment

To determine the effects of antisense oligodeoxynucleotide (ODN) treatment on CCD-induced thermal hyperalgesia, CCD and sham rats were treated with a spinal intrathecal administration of TRPV4 antisense ODN and mismatch ODN, respectively. The TRPV4 antisense ODN sequence, 5'-CATCACCAGGATCTGCCATACTG-3' (Invitrogen, Carlsbad, CA, USA) and the mismatch ODN sequence, 5'-CAACAGGAGGTTCCAGCAAACTG-3' (Invitrogen) were designed as described previously [2,3]. ODN was reconstituted in nuclease-free 0.9% NaCl (10  $\mu\text{g}/\mu\text{l}$ ) and administered into the spinal intrathecal space at a dose of 40  $\mu\text{g}$ , once a day for 7 days until the animals were sacrificed or treated with drugs. As described previously

[2,3], rats were anesthetized with 2.5% isoflurane inhalation anesthetic (97.5%  $\text{O}_2$ ), a 30-gauge needle was inserted into the subarachnoid space on the midline between the L4 and L5 vertebrae and 20  $\mu\text{l}$  ODN injected at 1  $\mu\text{l}/\text{s}$ , using a micro-syringe.

### 2.3. Western blot

Six DRGs from 3 rats dissected as one sample were quickly frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for further examination. Frozen tissues were homogenized in the homogenization buffer (50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 2 mM pepstatin A, 0.1% 2-mercaptoethanol). The crude homogenate was centrifuged at  $4^\circ\text{C}$  for 15 min at  $15,000 \times g$ . The supernatants were collected and measured three times. After quantification by BCA assay (Beyotime Biotech Inc., Jiangsu, People's Republic of China), 30  $\mu\text{g}$  of protein from each sample was separated by SDS-PAGE (12% gel) and transferred to polyvinyl fluoride membranes (Bio-Rad, Hercules, CA, USA). The nitrocellulose membrane was incubated in blocking buffer [5% non-fat dry milk in TTBS (100 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl and 0.1% Tween 20)] for 2 h at room temperature, followed by incubation overnight at  $4^\circ\text{C}$  in a polyclonal anti-TRPV4 preparation (1:500, Abcam, Cambridge, UK). The membranes were washed with blocking buffer to remove the non-specific binding and incubated with anti-rabbit IgG peroxidase conjugate (1:1500, Zhongshan Gold Bridge, Beijing, China) in blocking buffer for 1 h at room temperature. The membrane was then washed, incubated with and visualized by an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). The band intensities were quantified by AlphaEaseFC<sup>®</sup> Imaging software 4.0 (Alpha Innotech, San Leandro, CA, USA). The protein levels were expressed as a ratio of density of the detected band over that of  $\beta$ -actin (1:5000, Abcam, Cambridge, UK).

### 2.4. Behavioral testing

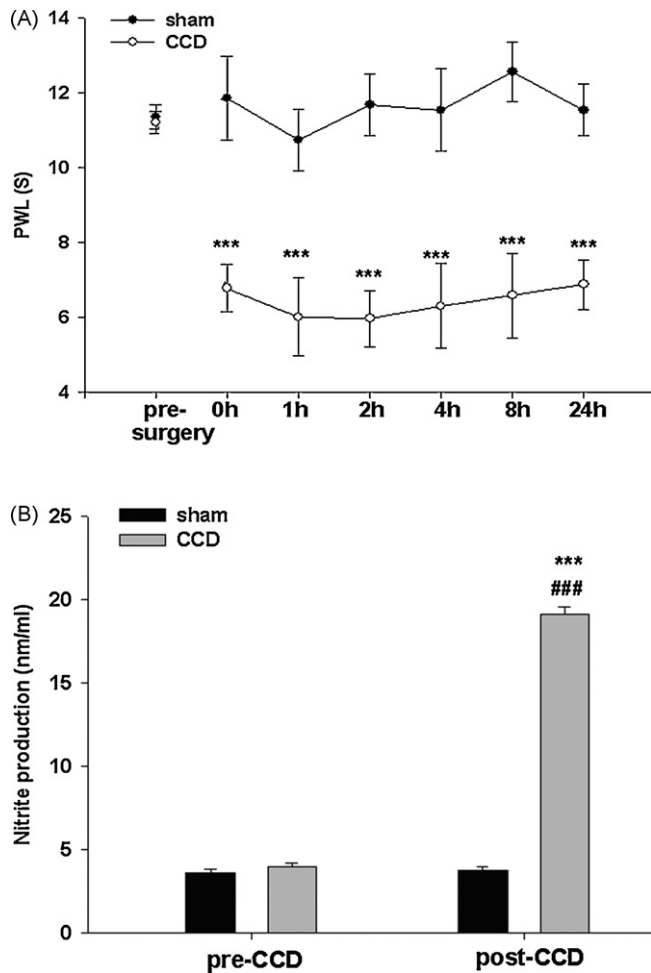
Thermal hyperalgesia was assessed using paw withdrawal latencies to radiant heat (BME-410A, Biomedical Engineering Institute of the Chinese Academy of Medical Sciences), as described previously [50]. Animals were acclimated to the cage with a 6-mm thick glass floor, and the temperature of the glass was measured and maintained at  $26 \pm 0.5^\circ\text{C}$ . Then, the radiant heat source beneath the glass floor was focused on the plantar surface of the ipsilateral hind paw when in contact with the glass floor. The paw withdrawal latencies per animal were obtained five times, with an intervening interval of 5 min. The intensity was pre-calibrated to give a baseline latency of approximately 10 s and the cutoff time was set at 20 s to avoid tissue damage. During this time, the rats initially demonstrated exploratory behavior, but subsequently stopped exploring and stood quietly with occasional bouts of grooming. The rats were tested on each of 2 successive days before surgery. Postoperative tests were conducted 2 h before chemicals treatment on the 7th day after surgery. Rats not demonstrating hyperalgesia were excluded from further study (less than 5%). Additional tests were conducted 1, 2, 4, 8, and 24 h after injection of chemicals or saline (2.5% DMSO) into the subarachnoid space on the midline between the L4 and L5 vertebrae on the 7th day after surgery. For experiments investigating the effect of treatment with TRPV4 antisense ODN on nociceptive thresholds, behavioral testing was performed 12 h after the last ODN injection. All behavioral tests were conducted under blind conditions.

### 2.5. Nitrite production assay

The level of nitrite as a measure of NO production in DRG was determined with modified Griess reagent. A nitrite detection kit (Beyotime Biotech Inc., Jiangsu, People's Republic of China) was used according to instructions provided by the manufacturer. In brief, the rats were decapitated 0, 1, 2, 4, 8, or 24 h after intrathecal injections of chemicals or 12 h after the last ODN injection. The L4 and L5 DRGs from two rats were pooled as one sample and each sample was homogenized in 300  $\mu\text{l}$  homogenization buffer (50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 2 mM pepstatin A, 0.1% 2-mercaptoethanol) and centrifuged at  $4^\circ\text{C}$  for 15 min at  $15,000 \times g$ , the supernatants were collected and assayed in triplicate, and a standard curve using  $\text{NaNO}_2$  was generated for each experiment for quantification. Briefly, 50  $\mu\text{l}$  of the supernatants or standard  $\text{NaNO}_2$  was mixed with 50  $\mu\text{l}$  of Griess reagent I and 50  $\mu\text{l}$  of Griess reagent II in a 96-well plate at room temperature for 10 min, and the absorbance was measured at 540 nm using a microplate reader. Results from three independent experiments were used for statistical analysis.

### 2.6. Chemicals and treatment

The following chemicals were used in this study:  $\text{N}^G$ -L-nitro-arginine methyl ester (L-NAME, inhibitor of NO synthase, Cayman, Ann Arbor, MI, USA),  $\text{N}^G$ -D-nitro-arginine methyl ester (D-NAME, an inactive isomer of L-NAME, Sigma, St. Louis, MO, USA), 1H-[1,2,4]-oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, a soluble guanylate cyclase (sGC) inhibitor, Cayman, Ann Arbor, MI, USA), the phorbol ester 4 $\alpha$ -phorbol 12,12-didecanoate (4 $\alpha$ -PDD, TRPV4 synthetic activator, Sigma), 8-(4-Chlorophenylthio) guanosine 3',5'-cyclic Monophosphothioate, Rp-Isomer sodium salt (Rp-8-pCPT-cGMPS, a PKG inhibitor, Sigma), and ruthenium red (TRPV4 antagonist, Sigma). All chemicals were dissolved in saline, with the exception of ODQ



**Fig. 1.** (A) Thermal hyperalgesia induced by chronic compression of the DRG (CCD) 7 days post-surgery.  $n=9$ . (B) Changes of NO metabolites nitrite in the DRG in CCD rats compared to sham operated rats 7 days post-CCD.  $n=9$  in each group. \*\*\* $P<0.001$  compared with the sham group. ### $P<0.001$  compared with corresponding pre-CCD. CCD: chronic compression of the dorsal root ganglion; DRG: dorsal root ganglion; NO: nitric oxide.

and 4 $\alpha$ -PDD, which were dissolved in 50% DMSO (Sigma). For all chemicals, final experimental dilutions were made in saline on the day of the experiment (final concentrations of DMSO were 2.5%). All these chemicals were injected intrathecally once in a 10  $\mu$ l volume followed by a 10  $\mu$ l saline flush. Doses and administration schedule of different inhibitors for intrathecal administration were selected based on previous reports [27,38,45] and on pilot experiments in our laboratory.

### 2.7. Statistical analysis

Statistical analysis was performed by one-way or two-way analysis of variance (ANOVA) followed by Newman–Keuls test or Tukey test when appropriate. Data were expressed as mean  $\pm$  standard error (S.E.M). A value of  $P<0.05$  was considered to be significant.

## 3. Results

### 3.1. Effects of antagonist and antisense ODN of TRPV4 on CCD-induced thermal hyperalgesia and levels of NO derivative nitrite in DRG

CCD produced a clear-cut hyperalgesia compared to the sham rats ( $F_{(1,112)} = 175.858$ ,  $P<0.001$ ) (Fig. 1A). The levels of NO derivative nitrite in DRG were significantly increased at 7 days in the CCD group when compared with the sham group ( $P<0.001$ ) (Fig. 1B). There were no significant changes in the levels of nitrite in the sham group ( $P=0.663$ ).

The intrathecal administration of the non-specific inhibitor of TRPV4 ruthenium red in concentrations of 0.1–1 nmol (both  $P<0.001$ ) produced a dose-dependent reduction of the thermal hyperalgesia in CCD rats, when compared with saline group, but ruthenium red in a concentration of 0.01 nmol did not ( $P=0.497$ ) (Fig. 2A). The significant reduction of the thermal hyperalgesia was observed at 1 h, peaked at 4 h, and lasted for about 8 h post-injection. There were no significant differences between groups at 24 h after administration (all  $P>0.05$ ). As shown in Fig. 2B, intrathecal injection of ruthenium red (1 nmol) significantly decreased the concentration of NO metabolites nitrite in DRG in CCD rats when compared to saline ( $P<0.001$ ). The reduction of nitrite in DRG peaked at 4 h and was positively associated with the changes of thermal responses ( $r=0.997$ ,  $P<0.05$ , Fig. 2A) after ruthenium red (1 nmol) injection at 7 days post-surgery.

Ruthenium red has inhibitory effects on TRPV4, TRPV1 [24], and intracellular stored  $Ca^{2+}$  release [57]. Due to the lack of specific pharmacological blockers of TRPV4, we used TRPV4 antisense ODN to interfere TRPV4 protein expression in the current study. Western blotting showed that, TRPV4 expression in DRG 7 days following compression was significantly ( $P<0.001$ ) inhibited by intrathecal injection of antisense ODN, but not inhibited by mismatch ODN treatment, as compared to controls (Fig. 2C and D).

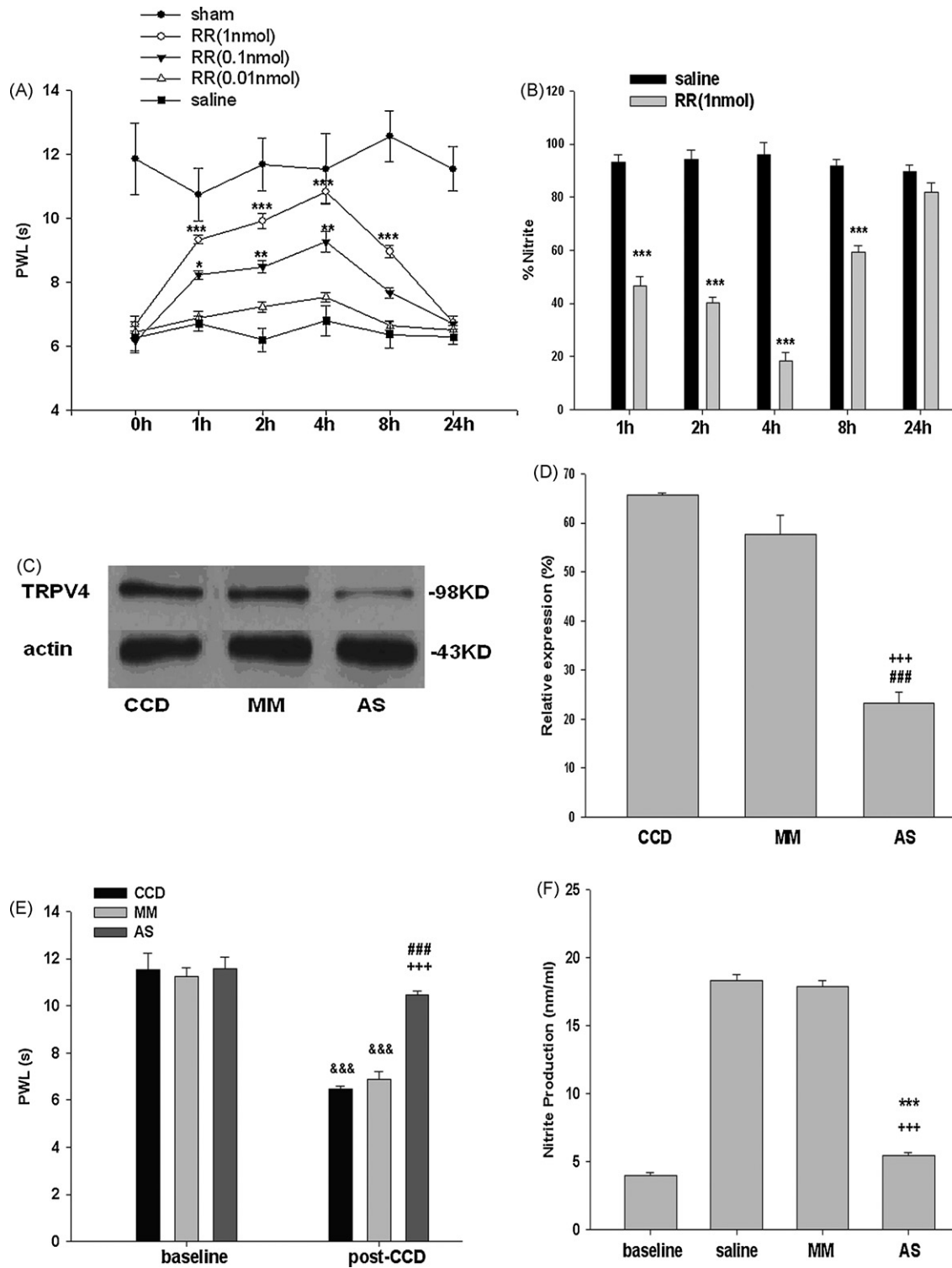
The PWLs in CCD rats were similar at baseline among groups (Fig. 2E, all  $P>0.05$ ), but after intrathecal ODN treatment for 7 days, the PWL was decreased significantly in the CCD and MM groups when compared with the baseline (both  $P<0.001$ ). In contrast, thermal hyperalgesia was partly reversed in the AS group ( $P=0.059$ ) compared with baseline. There were no significant changes in the PWL following intrathecal AS or MM in sham rats (data not shown). In CCD rats, the antisense ODN, but not the mismatch ODN, significantly ( $P<0.001$ ) inhibited the nitrite production when compared with the vehicle group (Fig. 2F).

### 3.2. Effects of L-NAME on CCD-induced thermal hyperalgesia and levels of NO derivative nitrite in DRG

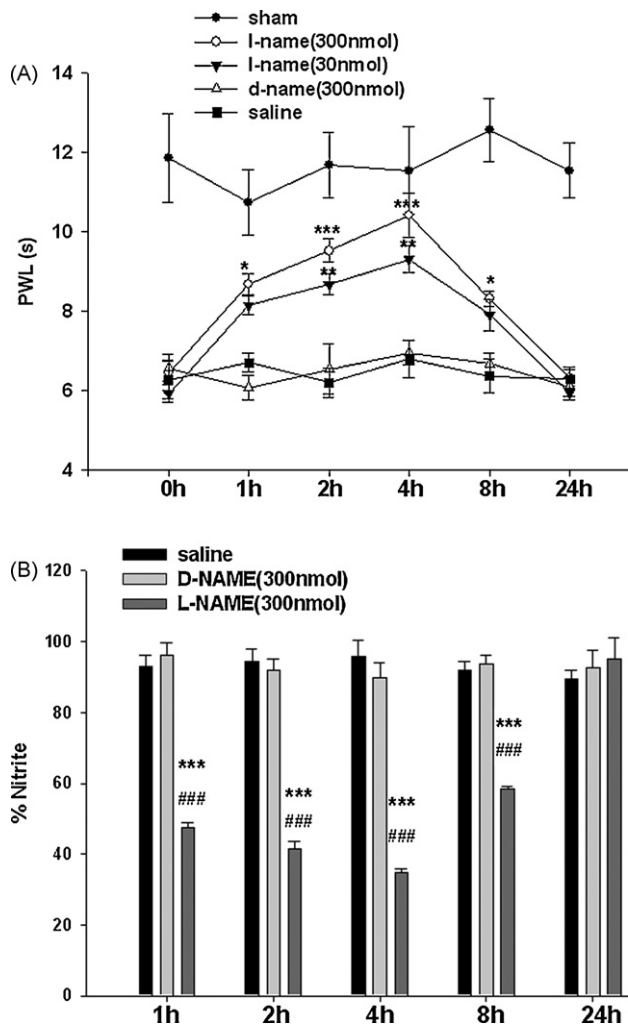
Intrathecal pretreatment with L-NAME (a non-specific NOS inhibitor, 30–300 nmol) significantly ( $P<0.001$ ) and dose-dependently inhibited CCD-induced thermal hyperalgesia from 1 to 8 h with the peak inhibitory effect at 4 h (Fig. 3A) post-injection. Moreover, as shown in Fig. 3B, the level of NO derivative nitrite in the DRG of CCD-treated rats was significantly ( $F_{(2, 120)} = 201.725$ ,  $P<0.001$ ) suppressed by intrathecal pretreatment with L-NAME (300 nmol). The level of nitrite began to decrease 1 h post-injection and reached a minimum 4 h post-injection, followed by a gradual recovery, and the changes in nitrite were not significantly different from the saline group at 24 h post-injection ( $P>0.05$ ). Moreover, intrathecal pretreatment with D-NAME (an inactive isomer of L-NAME, 300 nmol) had no effects on thermal hyperalgesia or on the concentration of nitrite at any time following DRG compression (both  $P>0.05$ , Fig. 3).

### 3.3. Effects of ODQ and Rp-8-pCPT-cGMPs on CCD-induced thermal hyperalgesia

The effects of ODQ (a sGC inhibitor, 50–100 nmol) and Rp-8-pCPT-cGMPs (a PKG inhibitor, 25–50 nmol) on CCD-induced thermal hyperalgesia are shown in Fig. 4. Intrathecal pretreatment with ODQ (50–100 nmol), or Rp-8-pCPT-cGMPs (25–50 nmol) significantly (both  $P<0.01$ ) and dose-dependently increased the PWL in CCD-treated rats 1, 2, 4 and 8 h post-injection when compared with vehicle treatment.



**Fig. 2.** (A) Dose-dependent inhibitory effects of RR (a TRPV4 antagonist) on CCD-induced thermal hyperalgesia. RR (0.01–1 nmol) were injected, respectively, 1 h after PWL evaluations and up to 24 h. Saline (0.9% NaCl) served as control. The sham group (black circle) served as a reference for the maximum possible effect.  $n = 9$  in each group. (B) The % nitrite in the DRG after intrathecal RR (1 nmol) treatment 1, 2, 4, 8 and 24 h post-injection. Saline (0.9% NaCl) served as control. % nitrite = (the level of nitrite after RR/saline treatment)/the level of nitrite 7 days post-CCD.  $n = 9$  in each group. (C) The Western blotting bands of the TRPV4 protein expression in CCD, MM and AS 7 days post-surgery.  $\beta$ -Actin served as an internal control. (D) TRPV4 relative expression (%) in CCD, MM, and AS group 7 days post-surgery.  $n = 3$ . (E) The effects of intrathecal TRPV4 ODN treatment on CCD-induced thermal hyperalgesia.  $n = 9$ . (F) The effect of TRPV4 ODN on the level of nitrite after 7 days post-CCD.  $n = 9$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with saline group. \*\*\*\* $P < 0.001$  compared with MM group. &&& $P < 0.001$  compared with corresponding pre-CCD. ### $P < 0.001$  compared with CCD group. RR: ruthenium red; CCD: chronic compression of the dorsal root ganglion; AS: antisense ODN; MM: mismatch ODN.



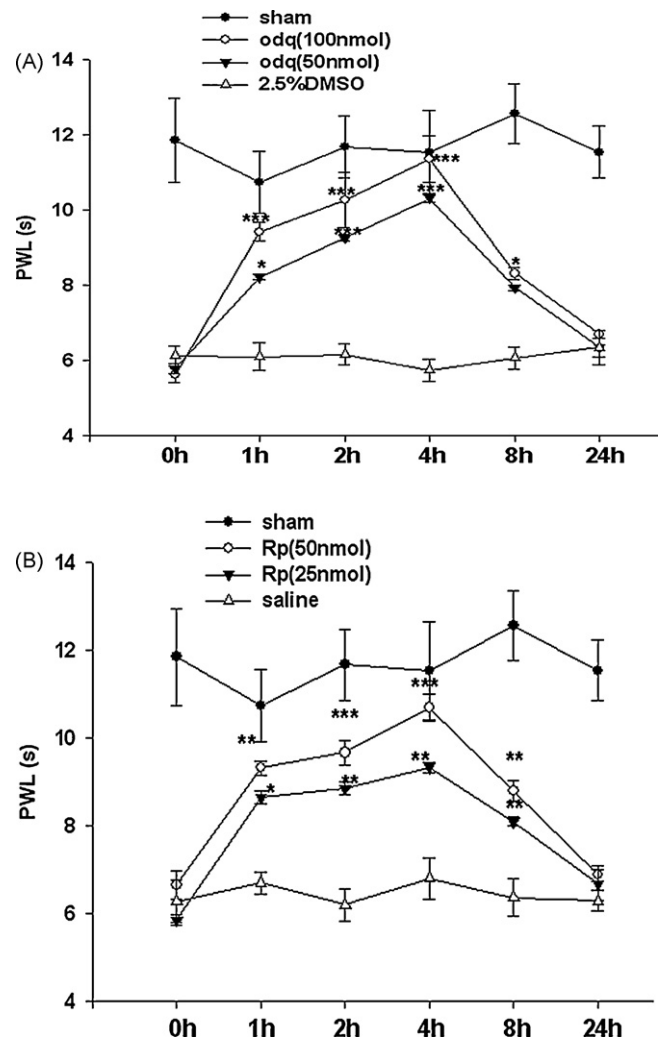
**Fig. 3.** (A) Dose-dependent inhibitory effects of L-NAME (30–300 nmol, a non-specific NOS inhibitor) on CCD-induced thermal hyperalgesia.  $n=9$ . (B) The % nitrite in the DRG after intrathecal L-NAME (300 nmol) treatment 1, 2, 4, 8 and 24 h post-injection. % nitrite = (the level of nitrite after L-NAME/D-NAME/saline treatment)/the level of nitrite 7 days post-CCD.  $n=9$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with saline group. ### $P < 0.001$  compared with D-NAME. CCD: chronic compression of the dorsal root ganglion; DRG: dorsal root ganglion.

#### 3.4. Effects of $4\alpha$ -PDD on the suppressive effects of L-NAME on CCD-induced thermal hyperalgesia and nitrite production

$4\alpha$ -PDD (the TRPV4 agonist, 1 nmol) attenuated the suppressive effects of L-NAME (300 nmol) on CCD-induced thermal hyperalgesia ( $P < 0.001$ ), compared to L-NAME (300 nmol) alone (Fig. 5A). Moreover, the decreased level of nitrite induced by intrathecal injection of L-NAME (300 nmol) was also attenuated by concomitant administration of  $4\alpha$ -PDD (1 nmol) 1 h post-injection ( $P < 0.05$ , Fig. 5B).

## 4. Discussion

This study demonstrates that the TRPV4-NO-cGMP-PKG pathway is involved in thermal hyperalgesia in CCD rats. Thermal hyperalgesia was partly attenuated by intrathecal blockers targeted at any site along this signal cascade. Inhibition of TRPV4 and/or NOS significantly decreased the nitrite production in DRG in CCD rats. Furthermore, changes in the level of nitrite are positively associated with the changes of thermal hyperalgesia. Finally,  $4\alpha$ -PDD (1 nmol), the TRPV4 agonist, attenuated the suppressive

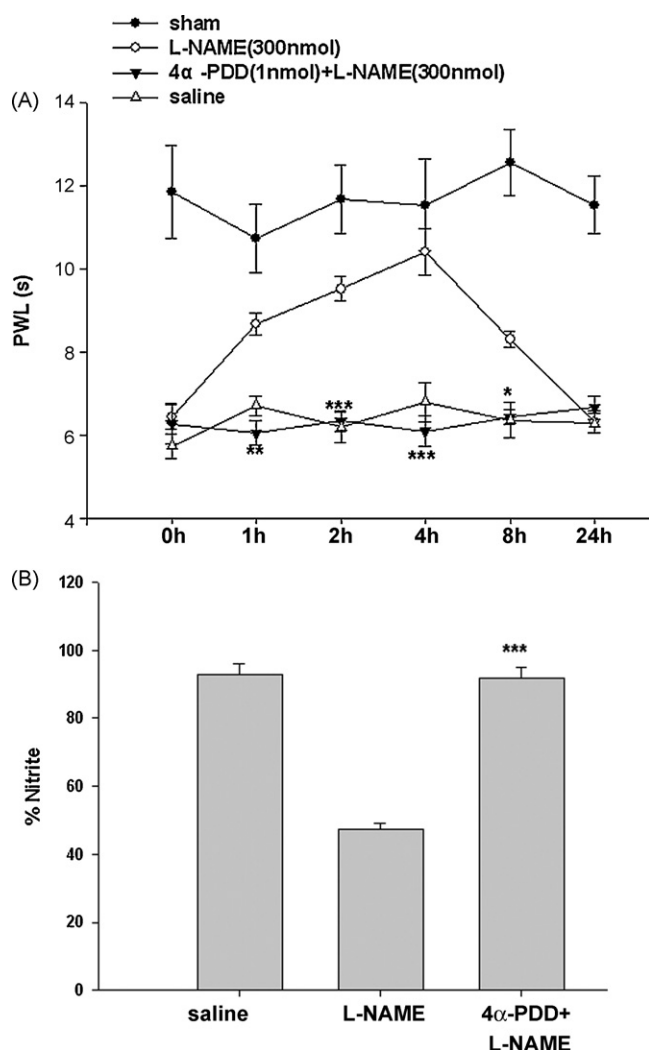


**Fig. 4.** (A) Dose-dependent inhibitory effects of ODQ (50–100 nm, a soluble guanylate cyclase inhibitor) on CCD-induced thermal hyperalgesia.  $n=9$ . (B) Dose-dependent inhibitory effects of Rp-8-pCPT-cGMPS (25–50 nmol, a PKG inhibitor) on CCD-induced thermal hyperalgesia.  $n=9$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with corresponding vehicle group. CCD: chronic compression of the dorsal root ganglion; Rp, Rp-8-pCPT-cGMPS; PKG: protein kinase G.

effects of L-NAME (300 nmol) on CCD-induced thermal hyperalgesia.

#### 4.1. TRPV4 as a thermal nociceptor in neuropathic pain

TRPV4 is a crucial mechano- or osmo-receptor in the DRG [28]. The TRPV4 channel is involved in neuropathic and inflammatory pain and plays an important pronociceptive role. It has been reported that inflammatory mediators might contribute to sensitization of the function of TRPV4, inducing pain-related behaviors and mechanical hypersensitivity [1,2]. Neuropathy-mediated sensitization of TRPV4 occurs during mechanical and hypotonic stimuli [3,4]. TRPV4 has been shown to contribute to CCD-induced mechanical hyperalgesia [59] and TRPV4 knockdown studies have shown ameliorated thermal hypersensitivity in inflammatory pathology [49]. Our results provide evidence that TRPV4 is also involved in the CCD-induced thermal hyperalgesia and may act as a thermo-nociceptor in neuropathic pain. Thus, TRPV4 may act as a mechano- and thermo-receptor in neuropathic and inflammatory pain.



**Fig. 5.** (A) Effects of 4 $\alpha$ -PDD (1 nm, the TRPV4 agonist) on the anti-thermal hyperalgesia effect of L-NAME (300 nmol) in CCD rats.  $n=9$ . (B) The % nitrite in the DRG after 1 h co-administration 4 $\alpha$ -PDD (1 nmol) with L-NAME (300 nmol). % nitrite = (the level of nitrite after 4 $\alpha$ -PDD + L-NAME/L-NAME/saline treatment)/the level of nitrite 7 days post-CCD.  $n=9$ . \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  compared with L-NAME group. CCD: chronic compression of the dorsal root ganglion; DRG: dorsal root ganglion.

#### 4.2. TRPV4-NO-cGMP-PKG pathway in neuropathic pain

Results in the current study show that TRPV4-induced NO production elicits sequential activation of soluble guanylyl cyclase and PKG to maintain thermal hypersensitivity following CCD. These results support our hypothesis that the TRPV4-NO-cGMP-PKG cascade is involved in neuropathic pain. The increased TRPV4 gene and protein expression [59], the increase in the level of NO production, the lack of increased NO production after inhibiting the function of TRPV4 by ruthenium red or antisense ODN, and the attenuated effects of 4 $\alpha$ -PDD on the analgesia and decreased nitrite production of the NOS blockers in CCD rats strongly suggest that the activation of TRPV4 contributes to the synthesis of NO. These results are supported by previous findings that 4 $\alpha$ -PDD and hypoosmotic stimulation could induce NO production, while ruthenium red, an inhibitor of TRPV4, inhibits the NO production [43]. The analgesic effects of ODQ and Rp-8-pCPT-cGMPS suggest that TRPV4-dependent NO production produces an increase in cGMP through the activation of sGC and succedent PKG, and this cascade has been reported in earlier reports [16,30]. The

synaptic transmission between DRG neurons and spinal dorsal horn neurons plays a key role in pain processing. It has been reported that the activation of TRPV4 enhances the synaptic transmission between DRG neurons and spinal dorsal horn neurons [8]. TRPV4 may modulate downstream effectors via glutamatergic synapses [7]. NO also contributes to glutamate release through a NO-sensitive sGC/cGMP-PKG signaling pathway on the rostral ventrolateral medulla neurons, which play an important role in nociceptive functions [19]. The involvement of NO/cGMP pathways in the modulation of glutamate release has also been demonstrated in slice preparations from the locus ceruleus [31]. On the other hand, NO inhibits the inhibitory neurotransmitter  $\gamma$ -aminobutyric acid (GABA)-evoked currents in dorsal root ganglion neuron via PKG-dependent pathways [5]. Further, TRPV4 contributes to CGRP release [17]. NO is also involved in CGRP synthesis and release. There is direct evidence that in DRG [9] and trigeminal ganglia neurons [12], an increase in the concentration of NO up-regulates CGRP synthesis and release, while treatment with a NOS inhibitor, asymmetric dimethylarginine, down-regulates CGRP level. It has been established that NO may sensitize the terminals of nociceptive primary afferent fibers and enhance neuropeptide release, possibly via mechanisms involving the activation of a cyclic guanidine monophosphate (cGMP)-dependent PKG [26]. NO also induces an increase in CGRP release from rat cranial dura mater [40], isolated aortic rings [6] or slices of spinal cords [15]. These evidence support the notion that TRPV4 contributes to NO synthesis [43], and NO may act through cGMP-PKG pathways to increase the release of glutamate and CGRP, which are mediators of pain transmission. Thus, there may be a close correlation between TRPV4 and NO-cGMP-PKG signaling pathways. Moreover, increasing evidence supports the idea that NO [23,34,46,47], NO-cGMP [32], cGMP-PKG [38], and NO-cGMP-PKG [45,48] play a facilitated role in nociceptive transmission in the pathologic pain states. Therefore, it is more likely that the activity of the TRPV4 channel in nociceptive DRG neurons in CCD could lead to intracellular  $Ca^{2+}$  influx, the increased expression of  $Ca^{2+}$ -dependent NOS, sequential increased NO-cGMP-PKG cascade response, and release of glutamate and CGRP. It is noteworthy that, the activation of spinal N-methyl-D-aspartate (NMDA) receptors and subsequent NO production have been considered to play a key role in generating acute and persistent pathological pain including neuropathic and inflammatory pain [16,25,56]. Further, stimulation of NMDA receptors leads to  $Ca^{2+}$ -mediated Arachidonic Acid (AA) release [11,54], and AA is an endogenous chemical activator of TRPV4 [13,53]. It is also reported that intrathecal administration of NMDA receptor antagonists d-2-amino-5-phosphonovaleric acid (APV) and dizocilpine maleate (MK-801) inhibits thermal hyperalgesia in CCD rats [37]. Therefore, it is possible that CCD-induced TRPV4-mediated  $Ca^{2+}$  influx in nociceptive DRG neurons may be associated with NMDA mediated AA release.

Given the partial reversal of TRPV4 related thermal hyperalgesia in CCD rats by blocking the pathway, other ionic and/or signal transduction mechanisms underlying pronociception can not be ruled out in the current study, such as: voltage-gated  $Na^+$  and  $K^+$  [20,44], hyperpolarization-activated cation current [55], PKA, PKC, PKD, phospholipase C $\beta$  [17], IP $_3$  [13], phospholipase A $_2$  [13], Src tyrosine kinase [1–4], and others [28].

In addition to the pronociceptive effect of the NO-cGMP-PKG pathway, this signal transmission pathway may also generate antinociceptive effects [42,52], which may have result from different effects of the NO-cGMP-PKG pathway in different subsets of nociceptive primary sensory neurons [51] and different upstream signals: NO released after activation of noradrenergic and muscarinic receptors generates analgesic effects [10,42]; in contrast, NO production after activation of NMDA receptors leads to hyperalgesic effects [16,25,56]. TRPV4-related thermal hyperalgesia in

the current study may be related to NO production after activation of NMDA receptors.

Three isoforms of NOS have been cloned: neuronal, endothelial and inducible NOSs [14]. The results of the current experiments did not allow us to determine which isoform of NOS was responsible for the TRPV4-induced NO synthesis, because L-NAME is a nonselective inhibitor of NOS [33]. Further studies are needed to identify the concrete isoform of NOS and the exact mechanism of the TRPV4-NO-cGMP-PKG pathway in the pronociceptive effect in chronic pain disorders.

It should be emphasized that our only evidence in the current study that this pathway operates in the DRG are the effects on measured nitrite levels in the DRG. All the other findings relied on intrathecal delivery of drugs that would affect the spinal cord as much or more than the DRG. We would have a better argument for a DRG locus if we had applied the drugs adjacent to the DRG, as has been done in another study [58].

In summary, TRPV4 enhances pain-related behavior through a NO-cGMP-PKG cascade that may occur within the DRG in CCD rats. The present results suggest that blockade of the TRPV4-NO-cGMP-PKG pathway could be an effective therapeutic approach for patients with chronic pain.

## Acknowledgments

This work is supported by the National Natural Science Foundation of China (grant No. 30472006 and 30872732) and the National Natural Science Foundation of Shandong Province China (no. Z2007C04). The technical assistance of Cardiovascular Research Center of Qilu Hospital and the Department of Pain Management, Shandong Provincial Hospital is gratefully acknowledged.

## References

- Alessandri-Haber N, Joseph E, Dina OA, Liedtke W, Levine JD. TRPV4 mediates pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediator. *Pain* 2005;118:70–9.
- Alessandri-Haber N, Yeh JJ, Boyd AE, Parada CA, Chen X, Reichling DB, et al. Hypotonicity induces TRPV4-mediated nociception in rat. *Neuron* 2003;39:497–511.
- Alessandri-Haber N, Dina OA, Yeh JJ, Parada CA, Reichling DB, Levine JD. Transient receptor potential vanilloid 4 is essential in chemotherapy-induced neuropathic pain in the rat. *J Neurosci* 2004;24:4444–52.
- Alessandri-Haber N, Dina OA, Joseph EK, Reichling DB, Levine JD. Interaction of transient receptor potential vanilloid 4, integrin, and SRC tyrosine kinase in mechanical hyperalgesia. *J Neurosci* 2008;28:1046–57.
- Bie BH, Zhao ZQ. Nitric oxide inhibits GABA-evoked current in dorsal root ganglion neuron via PKG-dependent pathway. *Brain Res Bull* 2001;55:335–9.
- Booth BP, Tabrizi-Fard MA, Fung H. Calcitonin gene-related peptide-dependent vascular relaxation of rat aorta. An additional mechanism for nitroglycerin. *Biochem Pharmacol* 2000;59:1603–9.
- Bourque CW, Ciura S, Trudel E, Stachniak TJ, Sharif-Naeini R. Neurophysiological characterization of mammalian osmosensitive neurones. *Exp Physiol* 2007;92:499–505.
- Cao DS, Yu SQ, Premkumar LS. Modulation of transient receptor potential Vanilloid 4-mediated membrane currents and synaptic transmission by protein kinase C. *Mol Pain* 2009;5:5.
- Chen QQ, Li D, Guo R, Luo D, Yang J, Hu CP, et al. Decrease in the synthesis and release of calcitonin gene-related peptide in dorsal root ganglia of spontaneously hypertensive rat: role of nitric oxide synthase inhibitors. *Eur J Pharmacol* 2008;596:132–7.
- Chen SR, Khan GM, Pan HL. Antiallodynic effect of intrathecal neostigmine is mediated by spinal nitric oxide in a rat model of diabetic neuropathic pain. *Anesthesiology* 2001;95:1007–12.
- Dumuis A, Sebben M, Haynes L, Pin JP, Bockaert J. NMDA receptors activate the arachidonic acid cascade system in striatal neurons. *Nature* 1988;336:68–70.
- Durham PL, Niemann C, Cady R. Repression of stimulated calcitonin gene-related peptide secretion by topiramate. *Headache* 2006;46:1291–5.
- Fernandes J, Lorenzo IM, Andrade YN, Garcia-Elias A, Serra SA, Fernandez-Fernandez JM, et al. IP3 sensitizes TRPV4 channel to the mechano- and osmotransducing messenger 5'-6'-epoxyeicosatrienoic acid. *J Gen Physiol* 2008;131:i2.
- Forstermann U, Kleinert H. Nitric oxide synthase: expression and expression control of the three isoforms. *Naunyn-Schmiedeberg Arch Pharmacol* 1995;352:351–64.
- Garry MG, Richardson JD, Hargreaves KM. Sodium nitroprusside evokes the release of immunoreactive calcitonin gene-related peptide and substance P from dorsal horn slices via nitric oxide-dependent and nitric oxide-independent mechanisms. *J Neurosci* 1994;14:4329–37.
- Garthwaite J, Charles SL, Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 1988;336:385–8.
- Grant AD, Cottrell GS, Amadesi S, Trevisani M, Nicoletti P, Materazzi S, et al. Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. *J Physiol* 2007;578:715–33.
- Hu SJ, Xing JL. An experimental model for chronic compression of dorsal root ganglion produced by intervertebral foramen stenosis in the rat. *Pain* 1998;77:15–23.
- Huang CC, Chan SH, Hsu KS. cGMP/protein kinase G-dependent potentiation of glutamatergic transmission induced by nitric oxide in immature rat rostral ventrolateral medulla neurons in vitro. *Mol Pharmacol* 2003;64:521–32.
- Huang ZJ, Song XJ. Differing alterations of sodium currents in small dorsal root ganglion neurons after ganglion compression and peripheral nerve injury. *Mol Pain* 2008;4:20.
- Liedtke W, Choe Y, Marti-Renom MA, Bell AM, Denis CS, Sali A, et al. Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell* 2000;103:525–35.
- Liedtke W, Friedman JM. Abnormal osmotic regulation in *trpv4*–/– mice. *Proc Natl Acad Sci USA* 2003;100:13698–703.
- Mabuchi T, Matsumura S, Okuda-Ashitaka E, Kitano T, Kojima H, Nagano T, et al. Attenuation of neuropathic pain by the nociceptin/orphanin FQ antagonist JTC-801 is mediated by inhibition of nitric oxide production. *Eur J Neurosci* 2003;17:1384–92.
- Mandadi S, Roufogalis BD. ThermoTRP Channels in Nociceptors: Taking a Lead from Capsaicin Receptor TRPV1. *Curr Neuropharmacol* 2008;6:21–38.
- Meller ST, Pechman PS, Gebhart GF, Maves TJ. Nitric oxide mediates the thermal hyperalgesia produced in a model of neuropathic pain in the rat. *Neuroscience* 1992;50:7–10.
- Miyasaka N, Hirata Y. Nitric oxide and inflammatory arthritides. *Life Sci* 1997;61:121–4.
- Ohkubo T, Shibata M, Takahashi H. The analgesia induced by intrathecal injection of ruthenium red. *Pain* 1993;54:219–21.
- O'Neil RG, Heller S. The mechanosensitive nature of TRPV channels. *Pflugers Arch* 2005;451:193–203.
- Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 1988;333:664–6.
- Paupardin-Tritsch D, Hammond C, Gerschenfeld HM, Nairn AC, Greengard P. cGMP-dependent protein kinase enhances Ca<sup>2+</sup> current and potentiates the serotonin-induced Ca<sup>2+</sup> current increase in snail neurones. *Nature* 1986;323:812–4.
- Pineda J, Kogan JH, Aghajanian GK. Nitric oxide and carbon monoxide activate locus coeruleus neurons through a cGMP-dependent protein kinase: involvement of a nonselective cationic channel. *J Neurosci* 1996;16:1389–99.
- Salter M, Strijbos PJ, Neale S, Duffy C, Follenfant RL, Garthwaite J. The nitric oxide-cyclic GMP pathway is required for nociceptive signalling at specific loci within the somatosensory pathway. *Neuroscience* 1996;73:649–55.
- Salvemini D, Wang ZQ, Wyatt PS, Bourdon DM, Marino MH, Manning PT, et al. Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br J Pharmacol* 1996;118:829–38.
- Sasaki A, Mabuchi T, Serizawa K, Takasaki I, Andoh T, Shiraki K, et al. Different roles of nitric oxide synthase-1 and -2 between herpetic and postherpetic allodynia in mice. *Neuroscience* 2007;150:459–66.
- Smith PL, Maloney KN, Pothren RG, Clardy J, Clapham DE. Bisandrographolide from *Andrographis paniculata* activates TRPV4 channels. *J Biol Chem* 2006;281:29897–904.
- Song XJ, Hu SJ, Greenquist KW, Zhang JM, LaMotte RH. Mechanical and thermal hyperalgesia and ectopic neuronal discharge after chronic compression of dorsal root ganglia. *J Neurophysiol* 1999;82:3347–58.
- Song XJ, Vizcarra C, Xu DS, Rupert RL, Wong ZN. Hyperalgesia and neural excitability following injuries to central and peripheral branches of axons and somata of dorsal root ganglion neurons. *J Neurophysiol* 2003;89:2185–93.
- Song XJ, Wang ZB, Gan Q, Walters ET. cAMP and cGMP contribute to sensory neuron hyperexcitability and hyperalgesia in rats with dorsal root ganglia compression. *J Neurophysiol* 2006;95:479–92.
- Song XJ, Zhang JM, Hu SJ, LaMotte RH. Somata of nerve-injured sensory neurons exhibit enhanced responses to inflammatory mediators. *Pain* 2003;104:701–9.
- Strecker T, Dux M, Messlinger K. Nitric oxide releases calcitonin gene-related peptide from rat dura mater encephali promoting increases in meningeal blood flow. *J Vasc Res* 2002;39:489–96.
- Suzuki M, Mizuno A, Kodaira K, Imai M. Impaired pressure sensation in mice lacking TRPV4. *J Biol Chem* 2003;278:22664–8.
- Takasu K, Honda M, Ono H, Tanabe M. Spinal alpha(2)-adrenergic and muscarinic receptors and the NO release cascade mediate supraspinally produced effectiveness of gabapentin at decreasing mechanical hypersensitivity in mice after partial nerve injury. *Br J Pharmacol* 2006;148:233–44.
- Takeda-Nakazawa H, Harada N, Shen J, Kubo N, Zenner HP, Yamashita T. Hyposmotic stimulation-induced nitric oxide production in outer hair cells of the guinea pig cochlea. *Hear Res* 2007;230:93–104.

- [44] Tan ZY, Donnelly DF, LaMotte RH. Effects of a chronic compression of the dorsal root ganglion on voltage-gated Na<sup>+</sup> and K<sup>+</sup> currents in cutaneous afferent neurons. *J Neurophysiol* 2006;95:1115–23.
- [45] Tanabe M, Nagatani Y, Saitoh K, Takasu K, Ono H. Pharmacological assessments of nitric oxide synthase isoforms and downstream diversity of NO signaling in the maintenance of thermal and mechanical hypersensitivity after peripheral nerve injury in mice. *Neuropharmacology* 2009;56:702–8.
- [46] Tanabe M, Sakaue A, Takasu K, Honda M, Ono H. Centrally mediated antihyperalgesic and antiallodynic effects of zonisamide following partial nerve injury in the mouse. *Naunyn Schmiedeberg Arch Pharmacol* 2005;372:107–14.
- [47] Tao F, Tao YX, Zhao C, Dore S, Liaw WJ, Raja SN, et al. Differential roles of neuronal and endothelial nitric oxide synthases during carrageenan-induced inflammatory hyperalgesia. *Neuroscience* 2004;128:421–30.
- [48] Tao YX, Hassan A, Haddad E, Johns RA. Expression and action of cyclic GMP-dependent protein kinase Ialpha in inflammatory hyperalgesia in rat spinal cord. *Neuroscience* 2000;95:525–33.
- [49] Todaka H, Taniguchi J, Satoh J, Mizuno A, Suzuki M. Warm temperature-sensitive transient receptor potential vanilloid 4 (TRPV4) plays an essential role in thermal hyperalgesia. *J Biol Chem* 2004;279:35133–8.
- [50] Villetti G, Bergamaschi M, Bassani F, Bolzoni PT, Maiorino M, Pietra C, et al. Antinociceptive activity of the N-methyl-D-aspartate receptor antagonist N-(2-Indanyl)-glycinamide hydrochloride (CHF3381) in experimental models of inflammatory and neuropathic pain. *J Pharmacol Exp Ther* 2003;306:804–14.
- [51] Vivancos GG, Parada CA, Ferreira SH. Opposite nociceptive effects of the arginine/NO/cGMP pathway stimulation in dermal and subcutaneous tissues. *Br J Pharmacol* 2003;138:1351–7.
- [52] Wang J, Zhang LC, Lv YW, Ji Y, Yan XJ, Xue JP. Involvement of the nitric oxide-cyclic GMP-protein kinase G-K<sup>+</sup> channel pathway in the antihyperalgesic effects of bovine lactoferrin in a model of neuropathic pain. *Brain Res* 2008;1209:1–7.
- [53] Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, Nilius B. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature* 2003;424:434–8.
- [54] Windelborn JA, Lipton P. Lysosomal release of cathepsins causes ischemic damage in the rat hippocampal slice and depends on NMDA-mediated calcium influx, arachidonic acid metabolism, and free radical production. *J Neurochem* 2008;106:56–69.
- [55] Yao H, Donnelly DF, Ma C, LaMotte RH. Upregulation of the hyperpolarization-activated cation current after chronic compression of the dorsal root ganglion. *J Neurosci* 2003;23:2069–74.
- [56] Yonehara N, Kudo C, Kamisaki Y. Involvement of NMDA-nitric oxide pathways in the development of tactile hypersensitivity evoked by the loose-ligation of inferior alveolar nerves in rats. *Brain Res* 2003;963:232–43.
- [57] Zhang BX, Ma X, Zhang W, Yeh CK, Lin A, Luo J, et al. Polyunsaturated fatty acids mobilize intracellular Ca<sup>2+</sup> in NT2 human teratocarcinoma cells by causing release of Ca<sup>2+</sup> from mitochondria. *Am J Physiol Cell Physiol* 2006;290:C1321–33.
- [58] Zhang JM, Homma Y, Ackerman WE, Brull SJ. Topical application of acidic bupivacaine to the lumbar ganglion induces mechanical hyperalgesia in the rat. *Anesth Analg* 2001;93:466–71.
- [59] Zhang Y, Wang YH, Ge HY, Arendt-Nielsen L, Wang R, Yue SW. A transient receptor potential vanilloid 4 contributes to mechanical allodynia following chronic compression of dorsal root ganglion in rats. *Neurosci Lett* 2008;432:222–7.