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ROLE OF PAR2 IN REGULATING OXALIPLATIN-INDUCED NEUROPATHIC PAIN VIA TRPA1

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Abstract

Oxaliplatin (OXL) is a third-generation chemotherapeutic agent commonly used to treat metastatic digestive tumors; however, one of the main limiting complications of OXL is neuropathic pain. In this study, the underlying mechanisms responsible for OXL evoked-neuropathic pain were examined. Using a rat model, the results demonstrated that intraperitoneal (i.p.) injection of OXL significantly increased mechanical pain and cold sensitivity as compared with control animals ($P < 0.05$ *vs.* control rats). Blocking proteinase-activated receptor 2 (PAR2) significantly attenuated mechanical pain and cold sensitivity observed in control rats and OXL rats ($P <$ 0.05 *vs.* vehicle control). The attenuating effect of PAR2 on mechanical pain and cold sensitivity were significantly smaller in OXL-rats than in control rats. The role played by PAR2 downstream signaling pathways [namely, transient receptor potential ankyrin 1 (TRPA1)] in regulating OXL evoked-neuropathic pain was also examined. The data shows that TRPA1 expression was upregulated in the lumbar dorsal root ganglion (DRG) of OXL rats and blocking TRPA1 inhibited mechanical pain and heightened cold sensitivity ($P < 0.05$ *vs.* control rats). Blocking PAR2 also significantly decreased TRPA1expression in the DRG. Findings in this study show that OXL intervention amplifies mechanical hyperalgesia and cold hypersensitivity and PAR2 plays an important role in regulating OXL-induced neuropathic pain via TRPA1 pathways.

Keywords

• oxaliplatin • neuropathic pain • Proteinase-activated receptor 2 (PAR2) • Transient receptor potential ankyrin 1 (TRPA1)

Introduction

One of the most common and distressing symptoms suffered by patients with progression of cancer is pain [1]. Cancer pain mainly arises from a tumor compressing or infiltrating nearby tissue; from skin, nerve and other changes caused by a hormone imbalance or immune response. Pain also arises from treatments and diagnostic procedures [1, 2]. It should be noted that chemotherapy and radiotherapy may produce painful conditions that persist long after treatment has ended [1, 3, 4]. Consequently, effective management of cancer pain related to these therapies becomes an important issue for treatment of cancer patients in clinics.

Oxaliplatin (OXL) is an organoplatinum compound and, as a third-generation chemotherapeutic agent, it is commonly used to treat cancer [5]. It has a significant activity against advanced and/or metastatic digestive tumors, but one of the main limiting complications of OXL

is painful neuropathy [6]. The signs of neuropathy start with paresthesia, followed by hyperesthesia [2]. Heightened cold sensitivity is another complication observed in cancer patients with OXL treatment [6]. Treatment options for these abnormal sensations have been restricted, partly due to a poor understanding of the underlying mechanisms responsible for neuropathic pain induced by chemotherapeutic agents such as OXL.

In a rat model, a single injection of OXL produces mechanical hyperalgesia and allodynia [7, 8]. OXL can induce mechanical hyperalgesia after initiation of the chemotherapy regimen in rats. The signs of mechanical hyperalgesia were ablated several weeks after discontinuation of OXL [7, 8]. In addition, the cold hypersensitivity was observed in animals with injection of OXL [7, 8]. This well established rat model, which is used to study the mechanisms of neuropathic pain induced by OXL, was employed in this study. Specifically examined were the effects

of proteinase-activated receptor 2 (PAR2) and transient receptor potential ankyrin 1 (TRPA1) in afferent nerves on mechanical and cold sensitivity in OXL-rats and control rats. The hypothesis was that injection of OXL increases PAR2 activation, which subsequently amplifies expression of TRPA1 receptor in the dorsal root ganglion (DRG) and thereby results in mechanical hyperalgesia and cold hypersensitivity.

Materials and methods

Experimental animals

All animal protocols were in accordance with the guidelines of the International Association for the Study of Pain and approved by the Research Administration Committee of this Hospital. Both genders of Wistar rats (200-250 g) were housed in individual cages with free access to food and water. The animals were kept in a temperature-controlled room (25℃) on a 12/12 h light/dark cycle.

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A model of neuropathic pain and administration of drugs

Oxaliplatin (Tocris Bioscience, R&D Systems, Minneapolis, MN, USA) was dissolved in a 5% glucose solution at a final concentration of 2 mg/ml. Acute neurotoxicity was induced in rats by a single intraperitoneal (i.p.) injection of oxaliplatin (6 mg/kg), as described previously [7, 8]. Control rats received the same volume of i.p. injection of vehicle. Mechanical allodynia and cold hypersensitivity were fully developed by OXL 3 days after injection.

PAR2 antagonist FSLLRY-NH2 (Tocris Bioscience, R&D Systems, Minneapolis, MN, USA) and TRPA1 antagonist HC030031 (Sigma-Aldrich, St. Louis, MO, USA) were injected i.p. and then mechanical and cold sensitivity were determined within 8 hrs after the drugs administration. The separated animals were used for experiments of the dosage response in each group.

Behavioral test

To quantify the mechanical sensitivity of the hindpaw, rats were placed in individual plastic boxes and allowed to acclimate for > 30 min. Mechanical paw withdrawal threshold (PWT) of rat hindpaw in response to the stimulation of von Frey filaments was determined. A series of calibrated von Frey filaments (ranging from 0.5 to 18.0 g) were applied perpendicularly to the plantar surface of the hindpaw with a sufficient force to bend the filaments for 60 s or until paw withdrew. In the presence of a response, the filament of next lower force was applied. In the absence of a response, the filament of next greater force was applied. To avoid injury during tests, the cutoff strength of the von Frey filament was 18 g. The tactile stimulus producing a 50% likelihood of withdrawal was determined using the "up-down" method [9]. Each trial was repeated 2 times at approximately 2 min intervals.

To examine cold sensitivity, Thermal Place Preference System (Coulbourn Instruments, Allentown, PA, USA) was used to perform the thermal place preference test in order to assess a cold avoidance behavior. Two connecting metal plates were surrounded by a plastic enclosure. The first plate was kept at neutral temperature (25°C) and the second plate was kept at cold temperature (12°C). The test was performed in darkness and each session lasted 3 minutes. During the session, the animals were left free to explore both plates. The time spent on the cold plate during the entire session was recorded using an infrared camera connected to a computer in order to determine cold avoidance behavior. To better control behavior test, the rats were repeatedly placed on the apparatus with both plates held at room temperature (25°C) for 3 minutes a few days before the beginning of the experiment. The rats spent an equal amount of time on each plate under these conditions, suggesting that animals showed no place preference. In addition, to avoid learning of any place preference unrelated to cold, the temperature of the plates were inverted between two consecutive sessions. Two trials were performed for each of the doses and data was averaged.

Western blot analysis

DRG tissues (L4-L6) were removed and total protein was extracted by homogenizing sample in ice-cold immunoprecipitation assay buffer. Lysates were centrifuged and the supernatants were collected. After being denatured, the supernatant samples containing 20 μg of protein were loaded onto gels and electrically transferred to a polyvinylidene fluoride membrane. The membrane was blocked and incubated overnight with primary mouse anti-TRPA1 antibodies (at dilution of 1:200, Cayman Chemical Company, Ann Arbor, MI, USA). Next, the membranes were washed and incubated with an alkaline phosphatase conjugated anti-mouse secondary antibody (1:1000). Chemiluminescence was used to detect the immunoreactive proteins. The bands recognized by the primary antibody were visualized by exposure of the membrane onto an x-ray film. Equal loading of the protein was shown by stripping the membrane and incubating it with mouse anti-β-actin. The film was scanned and the optical density of TRPA1 and β-actin bands was analyzed using the Scion Image software (Scion Corporation, Frederick, MD, USA).

Statistical analysis

All data was analyzed using a two-way repeated-measures analysis of variance (ANOVA). Values were presented as means ± SD. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed by using SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

OXL injection significantly decreased PWT as compared with glucose injection. PWT was 8.6 ± 1.9 g in control rats (n = 12) and 4.5 ± 1.2 g in OXL-rats (n = 12, $P < 0.05$ vs. control rats). Figure 1A shows that PWT was increased in a dose and time-dependent way after i.p. injection of FSLLRY-NH2 (0.01, 0.1, 1 mg/kg) in control rats and OXL-rats. As 1 mg/kg of FSLLRY-NH2 was given, its effects were observed 1 hr after its administration, peaked at 2 hrs and lasted for 6 hrs in control rats and for 3 hrs in OXL-rats. In addition, the percentage increase of PWT evoked by FSLLRY-NH2 was smaller in OXL-rats (n = 12) than that in control rats (n = 12). That is as 1mg/kg of FSLLRY-NH2 was given, PWT was increased by 47% in OXL-rats ($P < 0.05$ vs. control rats) and 78% in control rats 2 hrs after injection of FSLLRY-NH2. PWT was increased by 53% in OXL rats ($P < 0.05$ vs. control rats) and 71% in control rats 3 hrs after injection of FSLLRY-NH2.

Likewise, figure 1B showed that OXL injection significantly diminished the percentage of time spent on the cold plate as compared with glucose injection. The percentage time spend was 50 ± 10% in control rats (n = 12) and 32 ± 10% in OXL-rats (n = 12, $P < 0.05$ vs. control rats). Figure 1B further shows that blocking PAR2 in the sensory nerves by i.p. injection of FSLLRY-NH2 (1 mg/kg) significantly attenuated cold sensitivity to a greater degree in control rats (% time increased by 85%, n = 12) than in OXL-rats (% time increased by 43%, n = 12, $P < 0.05$).

The role played by TRPA1 in mediating PAR2 function of sensory nerves was also examined. Figure 2A showed that i.p. injection of TRPA1 antagonist, HC030031, attenuated mechanical sensitivity in OXL-rats and control rats as compared with vehicle injection. The inhibitory effects of HC030031 (1, 3, 10 mg/kg) on mechanical sensitivity appeared in a dose and time-dependent way and when 10 mg/kg of HC030031 was injected, its effects were
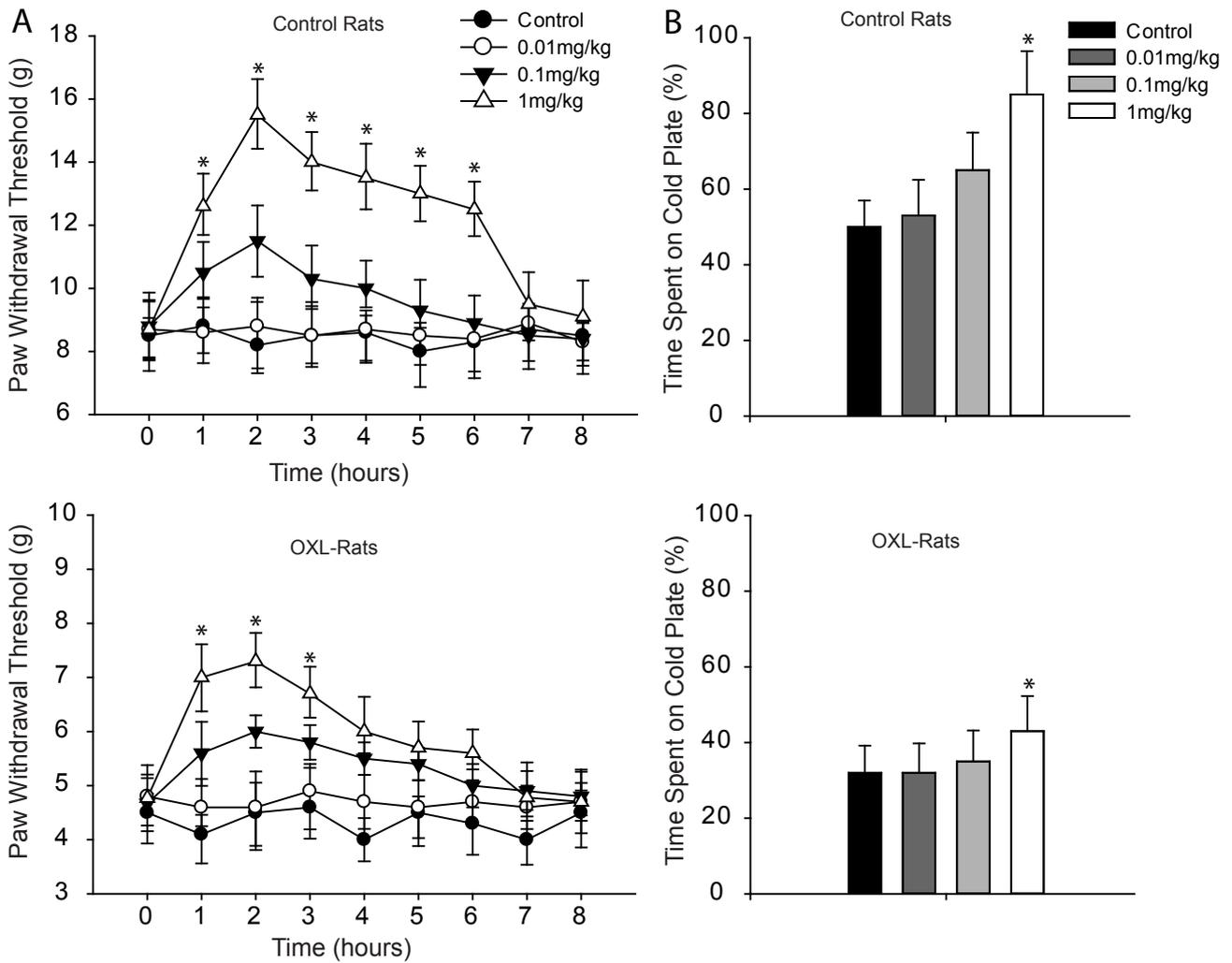
Figure 1. A. Effects of blocking PAR2 by administration of FSLLRY-NH2 on paw withdrawal threshold (PWT) in control rats and OXL-rats. As compared with vehicle injection, FSLLRY-NH2 increased PWT in control rats and OXL-rats. Note that the percentage increases of PWT evoked by attenuation of PAR2 were smaller in OXL-rats than that in control rats. B. Effects of blocking PAR2 in sensory nerves on cold sensitivity expressed as time spent on the cold plate (%) in control rats and OXL-rats. FSLLRY-NH2 elevated % time spent on the cold plates in control rats and OXL-rats, as compared with vehicle injection. In A and B, data are expressed as mean ± SD. *$P < 0.05$ vs. vehicle control and other dosages in A. *$P < 0.05$ vs. vehicle control and other dosages in control rats; and vs. vehicle control in OXL-rats in B. The number of rats = 6-12 in each group.

observed 1 hr after its administration, peaked at 2 hrs and lasted for 5 hrs in control rats and for 3 hrs in OXL-rats. The effects were smaller in OXL rats than in control rats ($P < 0.05$ vs. vehicle control, n = 10 in each group). In addition, figure 2B further showed that blocking TRPA1 in the sensory nerves by i.p. injection of HC030031 attenuated cold sensitivity to a greater degree in control rats (n = 12) than in OXL-rats (n = 12, $P < 0.05$, HC030031 vs. vehicle control for both control rats and OXL-rats).

A separated group of animals were used to examine expression of TRPA1. Figure 3

demonstrated that expression of TRPA1 was significantly increased in OXL-rats ($P < 0.05$ vs. control animals, n = 8) as compared with control rats (n = 6). Also, Figure 3 showed that FSLLRY-NH2 significantly decreased the amplified expression of TRPA1 induced by OXL ($P < 0.05$ vs. OXL-rats with vehicle, n = 10).

Discussion

Prior studies have shown that a single injection of OXL can induce neuropathic pain in rats, including mechanical hyperalgesia and cold

hypersensitivity [7, 8]. These abnormalities can be maintained for several days after OXL administration. Using the same intervention, in this study we observed significantly declined threshold (PWT) to evoke mechanical withdrawal and less time (%) spent on the cold plate three days after OXL injection (Figs. 1 and 2). This is consistent with the previous findings [7, 8, 10]. There was no significant difference observed in body weight gain between control rats and OXL-rats and no deterioration in general status was observed after injection of this dosage of OXL as reported previously
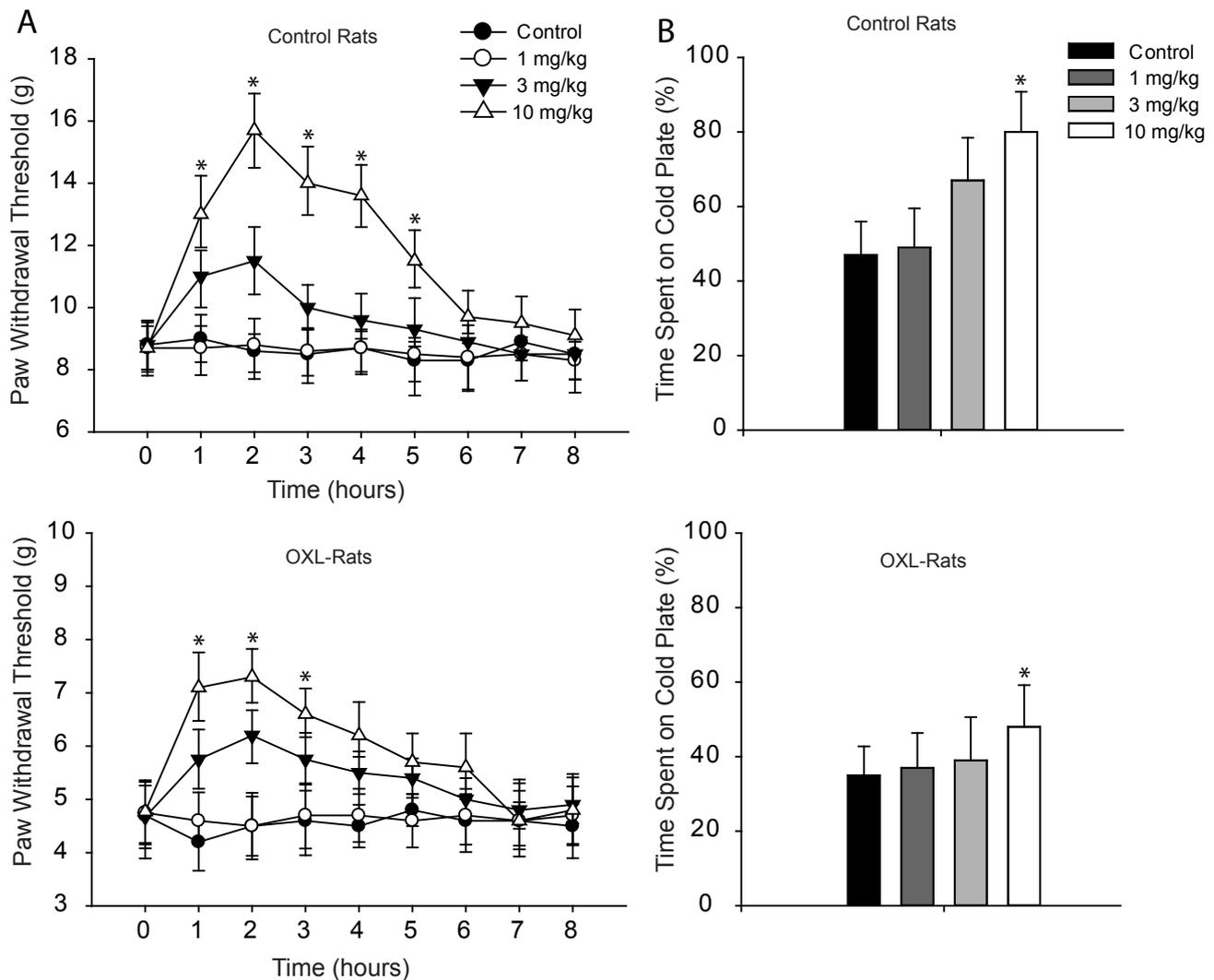
Figure 2. A. Effects of blocking TRPA1 by administration of HC030031 on paw withdrawal threshold (PWT) in control rats and OXL-rats. HC030031 increased PWT in control rats and OXL-rats as compared with vehicle injection, but the amplitude of PWT increases evoked by HC030031 was smaller in OXL-rats than that in control rats. B. Effects of blocking TRPA1 in sensory nerves on cold sensitivity expressed as time spent on the cold plate (%) in control rats and OXL-rats. HC030031 elevated % time spent on the cold plates in control rats and OXL-rats. In A&B, data are expressed as mean ± SD. *$P < 0.05$ *vs.* vehicle control and other dosages in A. *$P < 0.05$ *vs.* vehicle control and other dosages in control rats; and *vs.* vehicle in OXL-rats in B. The number of rats = 6-12 in each group.

[7, 8, 10]. The results further demonstrated that injection of PAR2 and TRPA1 antagonists significantly increased PWT and percent of time spent on the cold plate in control rats and OXL-rats, and the effects of blocking PAR2 and TRPA1 were significantly smaller in OXL-rats (Figs. 1A and 1B, and 2A and 2B). Consistently, the data of this study demonstrated that expression of TRPA1 protein was upregulated in the DRG neurons of OXL-rats as compared with control animals (Fig. 3). Interestingly, injection of FSLLRY-NH2 also significantly

attenuated enhanced TRPA1 expression in the DRG of OXL-rats (Fig. 3). Accordingly, the data in this study suggest that PAR2 and TRPA1 receptors in sensory nerves contribute OXL-induced mechanical hyperalgesia and cold hypersensitivity. We also suggest that the role played by PAR2 in regulating mechanical and cold sensitivity is via its downstream TRPA1 mechanisms.

PARs are a family member of G-protein-coupled receptors and are activated by a proteolytic mechanism [11]. Among the four

members of PARs, PAR2 is largely distributed in various tissues, including skin, gastrointestinal, cardiovascular, and respiratory systems. Of note, ~60% of DRG neurons at the L4-6 levels contain PAR2 [12, 13]. Stimulation of PAR2 by peripheral or central administration of non-inflammatory doses of PAR2 agonists evokes mechanical and thermal hyperalgesia in rodents [14, 15]. These studies further suggest that the releases of substance P and calcitonin gene-related peptide (CGRP) [14, 15] play a role in engagement of acute and chronic

pain by activation of PAR2. In experimental animal models, the expression of PAR2 is upregulated in the dorsal horn of the spinal cord after chemotherapy (e.g., paclitaxel) and blocking spinal PAR2 eliminates mechanical and thermal hyperalgesia observed in animals with paclitaxel [16]. Nevertheless, to the best of our knowledge it has not been reported that PAR2 pathways specifically contribute to OXL-induced hyperalgesia and the underlying mechanisms responsible for the role of PAR2 in regulating OXL-evoked neuropathic pain. In the present study, we suggest that the role played by PAR2 in regulating mechanical hyperalgesia and cold hypersensitivity is evoked by OXL.

TRPA1 has a functional role in pain and neurogenic inflammation resulting from channel activation to a variety of compounds including pungent agents, irritant chemicals, reactive oxygen and nitrogen species, and products of oxidative stress-induced lipid peroxidation [17-21]. TRPA1 has been shown to co-localize with TRPV1 in subpopulations of DRG neurons [20] and is engaged in development of bradykinin-induced mechanical hypersensitivity and painfully cold temperatures [22, 23]. Additional evidence supports the notion that TRPA1 mediates OXL-induced cold hypersensitivity [24]. Results of our current study further suggest that PAR2 plays an important role in regulating TRPA1 functions in OXL-evoked neuropathic pain because blocking PAR2 significantly attenuates the protein expression of TRPA1 in the DRG in engagement of OXL-evoked mechanical hyperalgesia and cold hypersensitivity.

The levels of numerous neurotransmitters and related receptors in sensory neurons-DRG neurons contribute to neuropathic pain [25, 26]. The DRG neurons supply primary afferent fibers and via DRG neurons the neurotransmitters release into the superficial dorsal horn of the spinal cord as the first synaptic site for pain transmission from peripheral afferent nerves to the central nervous system [26]. The levels of neurotransmitters, namely substance P and CGRP [27-29], in the DRG tissues are representative of their activities and the releases within the dorsal horn in regulating pain responses [26]. In addition, results of the prior studies by using animal models suggest a
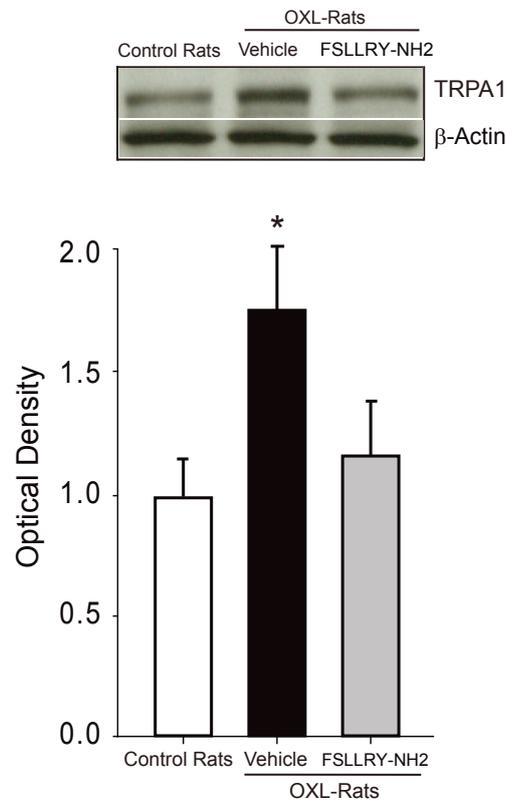

Figure 3. Top panels are typical bands and bottom panels are averaged data showing that TRPA1 expression in the dorsal root ganglion (DRG) tissues was upregulated in OXL-rats (n = 8) and FSLLRY-NH2 attenuated OXL-augmented TRPA1. Data are expressed as mean ± SD. *$P<0.05$ *vs.* control rats (n = 6) and OXL rats with FSLLRY-NH2 (n = 10). β-actin was used as equal loading control.

role for TRPA1 receptors in sensory nerves and/or at the spinal levels in regulating the releases of neurotransmitters including substance P and CGRP [30, 31]. Substance P and CGRP are two essential substrates considered to be responsible for common pain (i.e., due to inflammation and nerve damages etc.) or diabetes-induced neuropathic pain. We speculated that OXL would increase substance P and CGRP in the DRG and blocking respective PAR2 and TRPA1 receptors would attenuate the amplified substance P and CGRP.

In conclusion, inhibition of PAR2 and TRPA1 receptors in peripheral sensory nerves antagonizes mechanical hyperalgesia and cold hypersensitivity during OXL intervention. Protein expression of TRPA1 receptors are upregulated by injection of OXL. TRPA1 pathways play a role in PAR2 regulating OXL-induced neuropathic pain. Results of this

study will provide a base for the mechanisms responsible for chemotherapy (e.g., OXL)-induced neuropathic pain and further offer a strategy to target peripheral nerve system for treatment and management of neuropathic pain often observed in cancer patients. In addition, targeting one or more of these signaling molecules involved in activation of PAR2 and TRPA1 evoked by OXL may present new opportunities for treatment and management of neuropathic pain often observed in cancer patients.

Acknowledgment

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