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RAPID COMMUNICATION

TRPV1 Receptors Contribute to Paclitaxel-Induced c-Fos Expression in Spinal Cord Dorsal Horn Neurons

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Summary

Transient receptor potential vanilloid type 1 (TRPV1) receptors are important in the development of different pathological chronic pain states. Here we examined the role of spinal cord TRPV1 receptors in the mechanisms leading to activation of dorsal horn neurons after paclitaxel (PAC) treatment. PAC is a widely used chemotherapeutic drug that often leads to development of painful neuropathy. Immunohistochemical analysis of c-Fos protein expression in dorsal horn neurons was used as a marker of neuronal activation. Rat spinal cord slices were processed for *in vitro* incubation with PAC (100 nM) and TRPV1 receptor antagonists (SB366791 and AMG9810; 10 μM). PAC treatment induced significant upregulation of c-Fos nuclear expression in superficial dorsal horn neurons that was diminished by TRPV1 receptor antagonists pre-incubation. These results further substantiated the role of spinal TRPV1 receptors in the development of paclitaxel-induced neuropathic pain and contribute to better understanding of the pathological mechanisms involved.

Key words

c-Fos • Paclitaxel • TRPV1 • Neuropathy • Spinal cord

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Introduction

Paclitaxel (PAC) induced peripheral neuropathy (PIPN) and neuropathic pain syndromes are often a dose limiting adverse side effects of PAC anticancer therapy. For now, there is no effective treatment available, as the underlying cause of this PIPN remains poorly understood while many possible mechanisms were indicated (Boyette-Davis *et al.* 2015). Transient receptor potential vanilloid type 1 (TRPV1) receptors are expressed in the dorsal root ganglia (DRG) neurons and are known to act as integrators of noxious stimuli in the periphery (Nagy *et al.* 2014, Cui *et al.* 2016) and as modulators of nociceptive signaling in the spinal cord (Spicarova *et al.* 2014b). Recent studies suggested an active role of TRPV1 receptors in the pathological mechanisms of PIPN both in the peripheral and central nervous systems (Hara *et al.* 2013, Li *et al.* 2015). c-Fos is a small nuclear protein, translated from proto-oncogene *c-Fos* that has been widely used as a marker for activation of nociceptive neurons in the superficial laminae of spinal cord dorsal horn (Hunt *et al.* 1987). After *in vivo* paclitaxel treatment, low levels of PAC penetrates to the central nervous system (Yan *et al.* 2015). Here we tested hypothesis that application of low concentration PAC (100 nM) treatment would induce c-Fos expression in dorsal horn neurons that would be dependent on TRPV1 receptors activation. Immunohistochemical analysis was used to determine c-Fos expression in spinal cord slices incubated with PAC with and without the presence of TRPV1 antagonists SB366791 or AMG9810.

All experiments were approved by the Institutional Animal Care and Use committee and were carried out in accordance with the guidelines of the International Association for the Study of Pain and EU Directive 2010/63/EU for animal experiments. All efforts

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were made to minimize animal suffering and to reduce the number of animals used. Acute spinal cord slices were prepared altogether from 15 juvenile male Wistar rats ~ P21, as previously described (Spicarova *et al.* 2011, Spicarova *et al.* 2014a). The rats were deeply anesthetized (Forane ®, Abb Vie Czech Republic), lumbar spinal cords were removed and immersed in oxygenated ice-cold dissection solution containing (in mM): 95 NaCl, 1.8 KCl, 7 $MgSO_4$, 0.5 $CaCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$, 25 D-glucose, 50 sucrose. Acute transverse slices 350 µm thick were cut (Leica, VT 1200S, Germany) from the lumbar segments L4-L6, incubated for 30 min at 35 °C and then stored in incubating solution (in mM: 127 NaCl, 1.8 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, 25 D-glucose) at room temperature to recover for 1 h before the experiment. Alternating slices were assigned to the groups with different incubation protocol: paclitaxel (Sigma-Aldrich, PAC group, 100 nM, 60 min); TRPV1 antagonists only (SB366791 (Tocris Bioscience, UK), AMG9810 (Sigma-Aldrich), 10 µM, 70 min, groups SB and AMG respectively); TRPV1 antagonists (10 µM) for 10 min with added PAC (100 nM, 60 min) (SB+PAC or AMG+PAC groups). A control group of slices was incubated with vehicle (DMSO, 2 ‰, 70 min, CTRL group). Incubation solution was constantly saturated with 95 % O_2 and 5 % CO_2. Slices were fixed in 4 % paraformaldehyde, cryoprotected and cut in cryostat to 15-20 sections 16 µm thick from the middle portion of the slices. These sections were then immunohistochemically processed for detection of c-Fos expression. Briefly, sections were blocked with 3 % normal donkey serum, incubated overnight at 4 °C with anti-c-Fos (anti-rabbit, 1:2,000; Santa Cruz, USA) antibody in 1 % NDS with 0.3 % Triton X-100. For SABC staining, the sections were incubated for 2 h with biotinylated secondary antibody (1:400) and 2 h with peroxidase-conjugated streptavidin (1:400; Jackson ImmunoReasearch, USA). Finally, the reaction product was visualized with 1.85 mM DAB/0.003 % hydrogen peroxide in PBS for 2-5 min (Sigma-Aldrich, USA). Sections were photographed and analyzed using ImageJ software. Superficial laminae I/II of the spinal dorsal horn were outlined and measured (in pixels). Area and the number of immunoreactive neuronal nuclei for c-Fos in this region were counted. For every section the number of labeled neurons per measured area ratio was calculated. Cells/area (c/a) ratios were averaged for each slice and eight to sixteen slices from different rats were included in each experimental group. The data are represented as mean ± SEM. The difference between the groups was compared using One Way ANOVA followed by Holm-Sidak *post hoc* test (SigmaStat® software), the criterion for statistical significance was $P<0.05$.

To test the effect of PAC treatment on neuronal activation we measured the number of c-Fos-positive cells in the lumbar spinal cord slices. Our experiments showed that incubation for 1 h with 100 nM PAC increased significantly the number of c-Fos immunoreactive neurons in laminae I/II of the dorsal horn (Fig. 1; 2A, B, $P<0.001$). Pre-incubation with TRPV1 receptor antagonists in both experiments SB366791 (Fig. 2A, SB+PAC, $P<0.01$) and AMG9810 (Fig. 2B, AMG+PAC, $P<0.01$) significantly suppressed the PAC-induced c-Fos protein expression. The treatment with the AMG9810 alone, did not show any significant changes in the number of c-Fos expressing dorsal horn neurons from the CTRL group, while incubation with only SB366791 induced a minor increase in the c-Fos expression (Fig. 2A, SB, $P<0.05$).

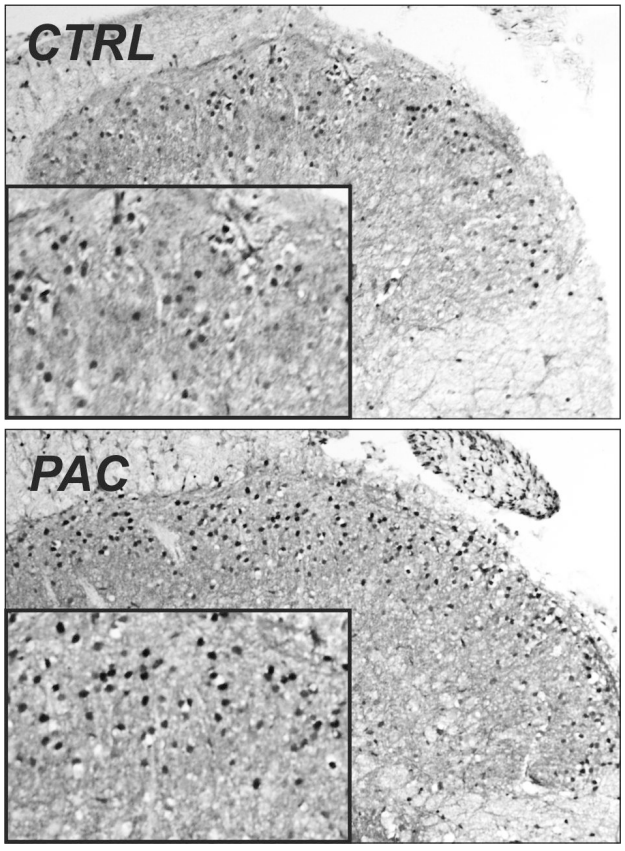

Fig. 1. Paclitaxel-induced upregulation of c-Fos expression in neuronal nuclei in the superficial area of spinal cord dorsal horn. Acute spinal cord slices were incubated with vehicle (CTRL) or with 100 nM paclitaxel (PAC). Inserts demonstrate c-Fos positive neuronal nuclei in the same slices at higher magnification.

A number of studies confirmed a wide diversity of peripheral stimuli that trigger upregulation of the immediate early gene *c-Fos* and its protein product c-Fos in dorsal horn neurons (Coggeshall 2005). However, in our *in vitro* study only central branches of primary afferents were present and thus the effect of PAC on spinal dorsal horn neurons had to be either due to the activation of presynaptic endings and/or due to action on the postsynaptic neurons. Recently it was shown that systemic administration of paclitaxel leads to thermal hypersensitivity and increased expression of TRPV1 receptors in DRG neurons (Hara *et al.* 2013). It was also documented (Li *et al.* 2015) that TRPV1 and TLR4 receptors are co-expressed in DRG neurons, intrathecal application of TRPV1 antagonist attenuated PAC-induced hypersensitivity and increase of TRPV1 expressing DRG neurons after PAC treatment was dependent on activation of Toll-like receptors 4 (TLR4). Additionally, PAC treatment increased TRPV1 receptors responsiveness to capsaicin also through TLR4 dependent pathway (Li *et al.* 2015). These findings suggest that in our study increase in c-Fos expression was mainly due to activation of presynaptic TLR4 and TRPV1 receptors. Activation of TLR4 receptors may beside others trigger *c-Fos* gene activation directly (Introna *et al.* 1986, Guha and Mackman 2001). However, in the spinal cord, TLR4 receptors are expressed predominantly in glial cells (Saito *et al.* 2010, Li *et al.* 2014) but not neurons (Li *et al.* 2014). Microglial activation could lead to release of cytokines and chemokines (Saito *et al.* 2010), potentiating presynaptic TRPV1 receptors function (Spicarova *et al.* 2011, Spicarova *et al.* 2014a). The effect of PAC treatment in this study was thus most likely mediated through presynaptic and/or glial TLR4 receptors followed by activation of presynaptic TRPV1 receptors, which led to increased release of neurotransmitters and neuromodulators such as glutamate, substance-P and calcitonin gene related peptide (CGRP) followed by activation of postsynaptic neurons and c-Fos expression. This is in agreement with increased spontaneous and miniature excitatory postsynaptic currents frequency after PAC treatment that was dependent on TRPV1 receptors activation in similar *in vitro* conditions (Li *et al.* 2015). While the effect of the TRPV1 antagonists in our experiments with c-Fos expression after low (100 nM) PAC treatment was robust, it is clear that other mechanisms play a role in the process (Boyette-Davis *et al.* 2015). Activation of protease activated receptor 2 (PAR2) and subsequently of protein kinases A, C and phospholipase C and sensitization of TRPV1, TRPV4 and TRP ankyrin 1 (TRPA1) was demonstrated to be important for PIPN pain development (Chen *et al.* 2011). High concentration (50 μM) PAC applied on esophagus slices induced CGRP release that was prevented by capsaicin induced desensitization and was reduced by TRPA1 and TRPV4 antagonists that also reduced mechanical allodynia present after systemic PAC treatment (Materazzi *et al.* 2012). In our experiments, both antagonists significantly diminished the PAC-induced c-Fos expression. However, application of SB366791 alone, but not AMG9810, induced a modest but still significant increase in c-Fos expression, when compared to the control group. This was most likely due to unspecific effect, while the two antagonists used exhibit different functional properties (Gavva *et al.* 2005).

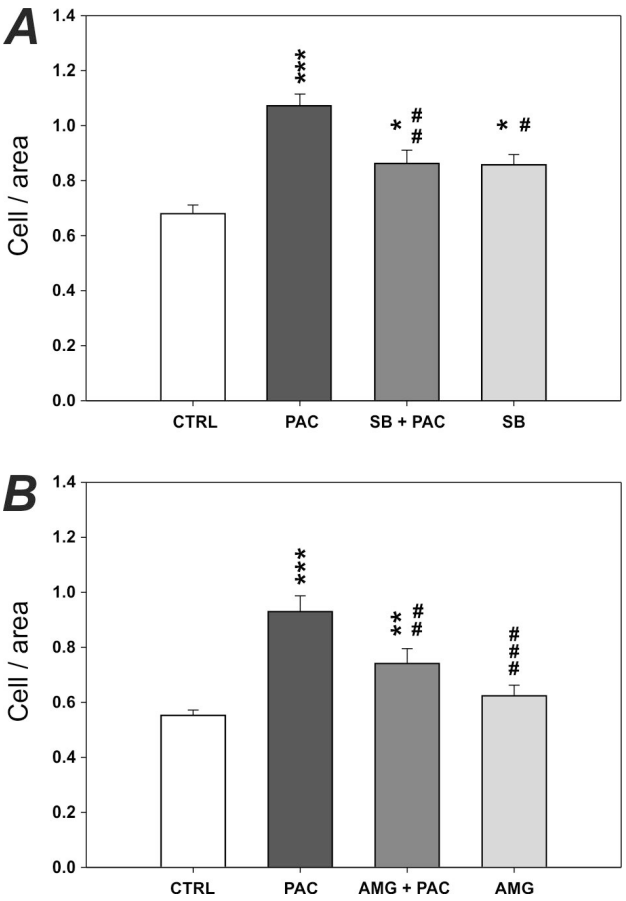

Fig. 2. The number of c-Fos-positive cells in the superficial spinal cord dorsal horn in controls (CTRL), after paclitaxel treatment (PAC), with TRPV1 antagonists added (SB+PAC; AMG+PAC) and TRPV1 antagonists only (SB; AMG). Data are presented as means ± SEM. Asterisks indicate significant difference from the control group (One Way ANOVA, Holm-Sidak *post hoc* test, * $P<0.05$; ** $P<0.01$; *** $P<0.001$). Hashtags indicate significant difference from the PAC group (One Way ANOVA, Holm-Sidak *post hoc* test, # $P<0.05$; ## $P<0.01$; ### $P<0.001$).

Our results showed that incubation of spinal cord slices *in vitro* with low concentration PAC induced c-Fos expression in dorsal horn neurons that was diminished by pre-treatment with TRPV1 antagonists. These findings further document the role of spinal cord TRPV1 receptors in the development of pathological pain states, including neuropathic pain after paclitaxel treatment.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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