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Upregulation of Nav1.3 Channel Induced by rrTNF in Cultured Adult Rat DRG Neurons *via* p38 MAPK and JNK Pathways

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Abstract

Activation of p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal protein kinase (JNK) in the dorsal root ganglia (DRG) is critical for the development of neuropathic pain. Tetraodontoxin-sensitive Nav1.3 channel, expressed at a very low level in the adult nervous system, is up-regulated in DRG neurons after peripheral nerve injury or peri-sciatic administration of rat recombinant tumour necrosis factor-alpha (rrTNF-α). To test if activation of p38 MAPK and JNK is required for the re-expression of Nav1.3 channel in cultured adult rat DRG neurons, we administrated rrTNF to cultured adult rat DRG neurons to induce Nav1.3 re-expression, and pre-treated with p38 MAPK inhibitor (SB203580 at 2.65, 26.5 and 265 μM) or JNK inhibitor (SP600125 at 1, 10 and 100 μM) 2 h before rrTNF to observe changes of Nav1.3-immunoreactivity. Compared with the DMSO vehicle pre-treatment group, SB203580 at 2.65 μM partially blocked the re-expression of Nav1.3 ($P < 0.001$), and at 26.5 and 265 μM completely blocked Nav1.3 ($P < 0.001$). Similarly, SP600125 at the concentration of 1 μM blocked the re-expression of Nav1.3 partially ($P < 0.001$), and at 10 and 100 μM blocked Nav1.3 completely ($P < 0.001$). These data show that the activation of both p38 MAPK and JNK in DRG neurons was involved in the re-expression of Nav1.3 channel triggered by TNF-α, which might contribute to neuropathic pain.

Key Words: tumour necrosis factor-α, p38 mitogen-activated protein kinase, c-Jun N-terminal protein kinase, sodium channel, neuropathic pain

Introduction

The expression of voltage-gated sodium channels in dorsal root ganglion (DRG) neurons, which are essential for the generation and propagation of action potentials, is believed to be responsible for neuropathic pain (29). Nav1.3, a subtype of tetraodontoxin-sensitive (TTX-S) sodium channel, is normally expressed at relatively high levels in embryonic nervous system and is barely detectable in the DRGs of adult rats (2, 35), but is re-expressed in DRG neurons after peripheral nerve injury (3, 11, 12, 21, 24, 35).

Tumour necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine, is generally believed to be an essential factor in the development of neuropathic pain. Our recent experiment has shown that selective injury of motor fibers by L5 ventral root transection (L5-VRT) up-regulates TNF-α, TNF receptor 1 (TNFR1) (40) as well as the expression of Nav1.3 (17) in uninjured L4 and L5 DRGs, and that inhibition of TNF-α synthesis prevents the re-expression of

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Nav1.3 (17) and neuropathic pain (40) induced by L5-VRT. Furthermore, peri-sciatic administration of rat recombinant TNF-α (rrTNF) without any nerve injury also produces neuropathic pain in bilateral hind paws and increases the expression of TNF-α and TNFR1 in ipsilateral L4 and L5 DRGs (36). Interestingly, peri-sciatic rrTNF induces a significant re-expression of Nav1.3 in uninjured L4 and L5 DRG neurons *in vivo*, and direct administration of rrTNF into cultured DRG neurons increases Nav1.3 expression *in vitro* (17) indicating that exogenous rrTNF may trigger the re-expression of Nav1.3 in DRG neurons by an autocrine mechanism contributing to the neuropathic pain. Therefore, the next work is to reveal the signal transduction mechanisms underlying the re-expression of Nav1.3 triggered by TNF-α.

A large body of studies has demonstrated that activations of p38 mitogen-activated protein kinase (p38 MAPK) (26, 27, 33, 39) and c-Jun N-terminal protein kinase (JNK) (14, 31, 42) in DRG or in spinal cord is required for the development of neuropathic pain. These pathways can be activated in neurons by a number of proinflammatory cytokines such as TNF-α (1, 9, 13, 38). After activation, JNK can phosphorylate several transcriptional factors such as c-Jun and induces gene transcription (6, 8). Meanwhile, activated p38 translocates into the nucleus and also initiates the transcription of a wide variety of target genes (19) most of which encode proteins that play an important and often determining role in the processes of immunity and inflammation. It has been shown that TNF induces phosphorylation of p38 MAPK and JNK in cultured DRG neurons (28). Whether or not the pathways of p38 MAPK and JNK participate in the mechanisms underling Nav1.3 re-expression triggered by TNF-α is still unknown. In the present work, we used rrTNF to induce the re-expression of Nav1.3 in cultured normal adult rat DRG neurons, and examined the effects of blockade of p38 MAPK or JNK pathway on the re-expression of Nav1.3 triggered by rrTNF *via* pre-treatment with p38 inhibitor (SB203580) or JNK inhibitor (SP600125) before rrTNF.

Materials and Methods

Subjects

A total of 50 male Sprague-Dawley rats, weighing 150 ± 20 g, were used. The rats were supplied by the Animal Experimental Center, Sun Yat-sen University, Guangdong, PRC (License number SCXK (yue) 2008-0002). The animals were housed in separated cages. The room temperature was kept at 24 ± 1°C and humidity 50-60% under a 12:12 light-dark cycle. The animals were allowed to access food and water *ad libitum*. All experimental procedures were approved by Animal Care Committee of Sun Yat-Sen University and were carried out in accordance with the guideline of the National Institutes of Health on animal care and the ethical guidelines for investigation of experimental pain in conscious animals (43).

Culturing of Adult Rat DRG Neurons

Dissociated cell culture of adult rat DRG neurons was performed as described in our recent work (17). Male Sprague-Dawley rats weighting 150 ± 20 g were used. Ganglias from the cervical to the lumbar level from 50 rats were excised and cut into pieces with a pair of scleral scissors. To avoid the potential effect of injury on the expression of Nav1.3, the operations were performed under low temperature (in a mixture of ice and water). After enzymatic and mechanical dissociation, the cells were seeded on poly-L-lysine (PL)-coated cover slips (Sigma, St. Louis, MO, USA) in 24-well plates in a humidified 95% air-5% CO_2 incubator (NuAire, Plymouth, MN, USA) for up to 4 days. In consistency with our previous works (17, 41), the cultured adult rat DRG neurons were allowed to extend neurites for about 3-4 days, which imitated "mature" DRG neurons.

Drug Treatments

Recombinant rat TNF-α (rrTNF) was stored as a stock solution of 10 μg/ml at –70°C, diluted to 100 pg/ml in 0.1% bovine serum albumin (BSA) in saline immediately before administration. SB203580, a p38 MAPK inhibitor, was first dissolved in dimethyl sulphoxide (DMSO) and then diluted with 0.9% NaCl to a final desired concentration of 2.65, 26.5 and 265 μM. SP600125, a JNK inhibitor, was diluted in the same way as SB203580 to 1, 10 and 100 μM (maximum final concentration of DMSO in both SB203580 and SP600125 were 0.2%, v/v).

After DRG neurons were incubated for 4 days, rrTNF at the concentration of 100 pg/ml was delivered into the cultured DRG neurons for 5 h to induce re-expression of Nav1.3-IR. In drug pre-treatment groups, p38 inhibitor SB203580 at 2.65, 26.5 and 265 μM, and JNK inhibitor SP600125 at 1, 10 and 100 μM, respectively, was applied 2 h before administration of rrTNF. In the vehicle group, 0.2% DMSO (vehicle of SB203580 and SP600125) was applied 2 h before rrTNF.

Immunocytochemistry

After drug treatments, the cultured neurons were fixed with 4% paraformaldehyde for 15 min, and

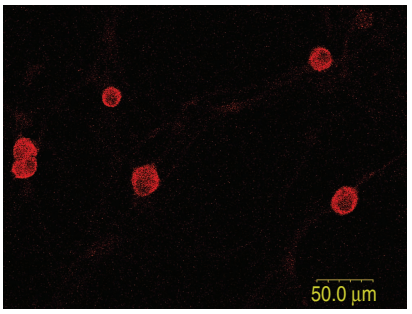

Fig. 1. Confocal image shows that administration of rrTNF to cultured adult DRG neurons induces re-expression of Nav1.3 (Immunocytochemistry staining, × 400).

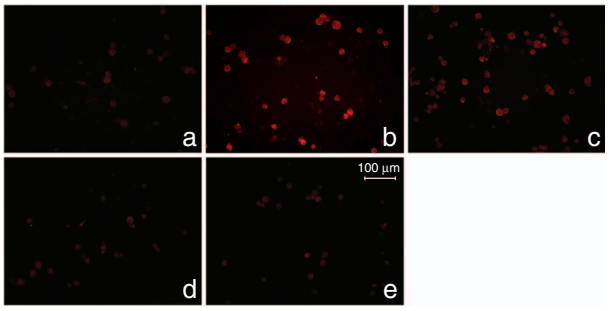

Fig. 2. p38 inhibitor SB203580 prevents the re-expression of Nav1.3 induced by rrTNF in cultured DRG neurons in a dose-dependent manner (Immunocytochemistry staining, × 200). (a) 0.1% BSA, vehicle of rrTNF, group; (b) 0.2% DMSO, vehicle of SB203580, pre-treated 2 h before rrTNF (100 pg/ml); (c-e) SB203580 at 2.65, 26.5 and 265 μM, respectively, pre-treated 2 h before rrTNF.

blocked with 3% donkey serum in 0.3% Triton X-100 for 1 h at room temperature, and then incubated overnight at 4°C with a rabbit anti-Nav1.3 antibody (Sigma, USA) at 1:300. After the primary antiserum incubation, the cells were incubated for 1 h at room temperature with Cy^3-conjugated donkey anti-rabbit IgG (1:300, Jackson ImmunoResearch, West Grove, PA, USA). The stained cells were mainly examined with a fluorescence microscope attached to a CCD spot camera (LEICA DFC350FX/DMIRB) and processed with LEICA IM50 software (Leica, Wetzlar, Germany). Some cells were examined using the Olympus FV500 confocal microscope. Cells were optically sectioned in the xy plane with a minimum slice thickness of 0.5 μm with multiple scan averaging. Images were taken through the central planes of all cells and processed with Adobe Photoshop.

To verify the specificity of the Nav1.3 antiserum, a preincubation method was used as in our recently works (17, 41) and only background levels of fluorescence were detected in the negative control experiments incubated the without primary antibody (data not shown).

Image Analysis

The immunoreactivity (IR) of Nav1.3 in cultured DRG neurons was analyzed using the LEICA Qwin V3 digital-image processing system (Leica, Wetzlar, Germany). For analysis of each group, the average and standard deviation of Nav1.3-IR density were obtained by measuring the optical densities of 400 neurons in ten or twelve randomly selected images, and then normalized relatively to the value of the vehicle group.

Statistical Analysis

Statistical analysis was performed with SPSS 10.0 (SPSS Inc., Chicago, Illinois, USA). All data was presented as means ± SD. Differences in changes of values over doses were tested using one-way ANOVA followed by individual post hoc comparisons (Tukey post hoc tests) or using Student's *t*-test if only two groups were applied. A value of $P < 0.05$ was considered statistically significant.

Results

Blockade of p38 MAPK Pathway Inhibits Re-Expression of Nav1.3 Triggered by rrTNF

Our recent work has confirmed that rrTNF at the concentrations of 10, 100 and 1,000 pg/ml but not at 1 pg/ml increased the expression of Nav1.3-IR in cultured DRG neurons 5 h after administration (17). In the present work, rrTNF at the concentration of 100 pg/ml was used to induce re-expression of Nav1.3-IR. To elucidate the roles of p38 MAPK in Nav1.3 re-expression in cultured DRG neurons, p38 inhibitor SB203580 at 2.65, 26.5 and 265 μM, respectively, was applied 2 h before rrTNF. In the vehicle of the SB203580 group, 0.2% DMSO was applied 2 h before rrTNF. As shown in Fig. 1, administration of rrTNF, but not the vehicle (0.1% BSA, Fig. 2a), to cultured DRG neurons significantly increased the expression of Nav1.3. After pre-treatment with 0.2% DMSO, no effect was observed on Nav1.3 re-expression triggered by rrTNF (Fig. 2b). Nav1.3-IR density in DMSO pre-treatment group was still significantly higher than that in the BSA vehicle group ($P < 0.001$, Fig. 3). SB203580 at the concentration of 2.65 μM partially blocked the re-expression of Nav1.3 (Figs. 2, b and c), and at 26.5 and 265 μM blocked Nav1.3 completely (Figs. 2, b, d and e). As shown in Fig. 3, the density of Nav1.3-IR in the 2.65 μM SB203580 pre-treatment group was lower than that in the DMSO pre-treatment group ($P < 0.001$) and higher than that in the BSA

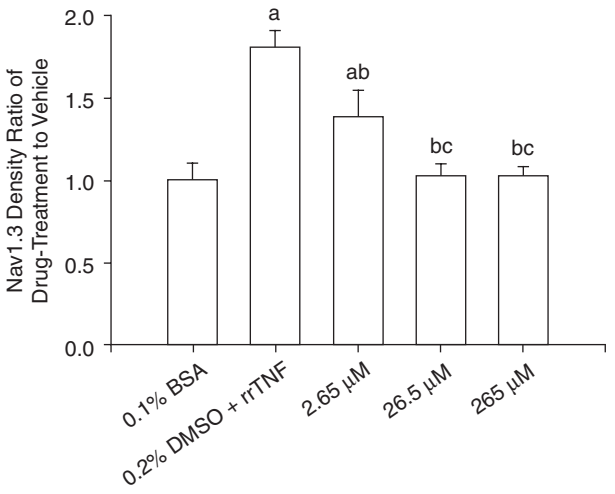

Fig. 3. Quantitative analysis of Nav1.3-IR density in SB203580 pre-treatment DRG neurons. 0.1% BSA: vehicle of rrTNF group; 0.2% DMSO + rrTNF: vehicle of SB203580 pre-treated 2 h before rrTNF; 2.65, 26.5 and 265 μM: SB203580 at 2.65, 26.5 and 265 μM, respectively, pre-treated 2 h before rrTNF; Each bar is expressed as the means ± SD. $^{a}P < 0.001$, *vs*. the BSA vehicle group; $^{b}P < 0.001$, *vs*. the DMSO pre-treatment group; $^{c}P < 0.001$, *vs*. the 1 μg/ml SB203580 pre-treatment group.

vehicle group ($P < 0.001$). Nav1.3-IR densities in both 26.5 and 265 μM SB203580 pre-treatment groups were not different from that in the BSA vehicle group ($P > 0.05$ *vs*. the BSA vehicle group; $P < 0.001$ *vs*. the DMSO pre-treatment group).

Blockade of JNK Pathway Inhibits Re-Expression of Nav1.3 Triggered by rrTNF

To evaluate the role of JNK pathway in Nav1.3 re-expression in cultured DRG neurons, JNK inhibitor SP600125 at 1, 10 and 100 μM, respectively, was applied 2 h before rrTNF. As shown in Fig. 4, SP600125 at the concentration of 1 μM partially blocked Nav1.3 re-expression, and at 10 and 100 μM blocked Nav1.3 expression completely. As shown in Fig. 5, the Nav1.3-IR density in the 1 μM SP600125 pre-treatment group was lower than that in the DMSO pre-treatment group ($P < 0.001$) and higher than that in the BSA vehicle group ($P < 0.001$). Nav1.3-IR densities in both the 10 and 100 μM SP600125 groups were not different from that in the BSA vehicle group ($P > 0.05$ *vs*. the BSA vehicle group; $P < 0.001$ *vs*. the DMSO pre-treatment group).

Discussion

In the present *in vitro* study, the re-expression of Nav1.3 induced by rrTNF in cultured adult rat DRG neurons was blocked by pre-treatment with

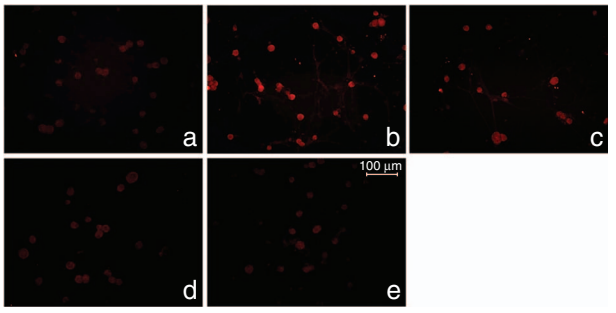

Fig. 4. JNK inhibitor SP600125 prevents the re-expression of Nav1.3 induced by rrTNF in cultured DRG neurons in a dose-dependent manner (Immunocytochemistry staining, × 200). (a) 0.1% BSA, vehicle of rrTNF, group; (b) 0.2% DMSO, vehicle of SP600125, pre-treated 2 h before rrTNF (100 pg/ml); (c-e) SP600125 at 1 μM, 10 μM and 100 μM, respectively, pre-treated 2 h before rrTNF.

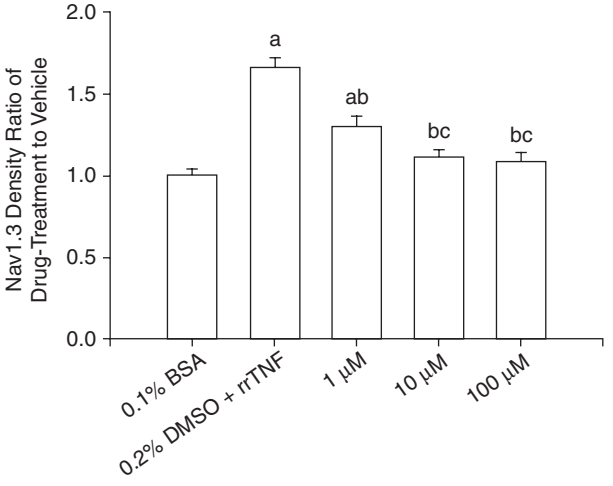

Fig. 5. Quantitative analysis of Nav1.3-IR density in SP600125 pre-treatment DRG neurons. 0.1% BSA: vehicle of rrTNF group; 0.2% DMSO + rrTNF: vehicle of SP600125 pre-treated 2 h before rrTNF; 1 μM, 10 μM, 100 μM: SP600125 at 1 μM, 10 μM and 100 μM, respectively, pre-treated 2 h before rrTNF; Each bar is expressed as the means ± SD. $^{a}P < 0.001$, *vs*. the BSA vehicle group; $^{b}P < 0.001$, *vs*. the DMSO pre-treatment group; $^{c}P < 0.001$, *vs*. the 1 μM SP600125 pre-treatment group.

p38 MAPK inhibitor SB203580 or JNK inhibitor SP600125 in a dose-dependent manner suggested that the activation of p38 MAPK and JNK was involved in the re-expression of Nav1.3 triggered by TNF-α, which might contribute to neuropathic pain.

It is well established that peripheral nerve injury induces neuropathic pain by triggering abnormal spontaneous discharges of action potentials (ectopic discharges) (15, 34), which occur in both injured site and dorsal root ganglion (DRG) (5, 10). At present,

it is generally accepted that abnormal expression of Nav1.3 channels may play an important role in the generation of ectopic discharge, as the channel mediates a TTX-S current with fast activation and inactivation kinetics and rapid recovery from inactivation leading to reduction of firing threshold and/or relatively high frequency discharge (7). Hains *et al*. (16) have reported that inhibition of Nav1.3 re-expression by intrathecal injection of Nav1.3-specific antisense oligodeoxynucleotides attenuates mechanical allodynia and thermal hyperalgesia produced by peripheral nerve injury and reduces the hyperresponsiveness of dorsal horn neurons. Our recent work has also shown that selective injury of motor fibers but leaving sensory neurons intact by L5-VRT, which leads to neuropathic pain (23, 40), induces the re-expression of Nav1.3 at both mRNA and protein levels in uninjured L4 and L5 DRGs, and the time course of the channel expression is parallel to the behavioral signs of neuropathic pain (17). More interestingly, we found that increased TNF-α in uninjured DRG neurons is responsible for the re-expression of Nav1.3 (17). Up to date, however, the signal transduction mechanism underlying the re-expression of Nav1.3 triggered by TNF-α is still unknown.

There are reports that TNF-α activates p38 (30, 32) or JNK (13, 38) cascade to trigger pain behaviors. Both peripheral nerve injury (20, 22) and selective motor fibers injury by L5-VRT (39) result in the activation of p38 in L5 spinal cord and L5 DRG. blockade of TNF-α synthesis prevents the upregulation of phospho-p38 in uninjured DRG neurons and spinal dorsal horn (39). The use of different p38 inhibitors alleviates the abnormal pain behaviors induced by nerve injury (18, 20, 30, 37, 39). Furthermore, JNK, another member of the MAPK family, is persistently activated in spinal cord (25, 42) and DRG neurons (26) after nerve injury. Different methods used to inhibit the activation of JNK can attenuate the neuropathic pain (26, 42). Recently, Black *et al*. (4) have reported that both Nav1.3 channels and activated p38 are upregulated in axons within human painful neuromas. Our previous work has also shown that perisciatic administration of rrTNF without any nerve injury leads to mechanical allodynia in bilateral hindpaws lasting for about 20 days (36). Exogenous rrTNF increases the expression of TNF-α and TNFR1 in ipsilateral L4 and L5 DRGs (36), and upregulates Nav1.3 channels in uninjured DRG neurons *in vivo* and *in vitro* (17). In the present *in vitro* work, we confirmed again that directly administration of rrTNF to cultured adult rat DRG neurons induced re-expression of Nav1.3 channels, and found that blockade of the downstream signal molecules of TNF-α, including p38 MAPK and JNK, prevented the re-expression of Nav1.3 triggered by rrTNF in a dose-dependent manner. Taken together, the activation of p38 MAPK and JNK was involved in the re-expression of Nav1.3 triggered by TNF-α, which might contribute to neuropathic pain.

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