Neuroscience 166 (2010) 1–4

NEURON-RESTRICTIVE SILENCER FACTOR CAUSES EPIGENETIC SILENCING OF $K_V4.3$ GENE AFTER PERIPHERAL NERVE INJURY

H. UCHIDA,[1] K. SASAKI,[1] L. MA AND H. UEDA*

Division of Molecular Pharmacology and Neuroscience, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

Abstract—Peripheral nerve injury causes a variety of alterations in pain-related gene expression in primary afferent, which underlie the neuronal plasticity in neuropathic pain. One of the characteristic alterations is a long-lasting downregulation of voltage-gated potassium (K_v) channel, including $K_v4.3$, in the dorsal root ganglion (DRG). The present study showed that nerve injury reduces the messenger RNA (mRNA) expression level of $K_v4.3$ gene, which contains a conserved neuron-restrictive silencer element (NRSE), a binding site for neuron-restrictive silencer factor (NRSF). Moreover, we found that injury causes an increase in direct NRSF binding to $K_v4.3$-NRSE in the DRG, using chromatin immunoprecipitation (ChIP) assay. ChIP assay further revealed that acetylation of histone H4, but not H3, at $K_v4.3$-NRSE is markedly reduced at day 7 post-injury. Finally, the injury-induced $K_v4.3$ downregulation was significantly blocked by antisense-knockdown of NRSF. Taken together, these data suggest that nerve injury causes an epigenetic silencing of $K_v4.3$ gene mediated through transcriptional suppressor NRSF in the DRG. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: neuropathic pain, dorsal root ganglion, transcription, epigenetic, voltage-gated potassium channel, neuron-restrictive silencer factor.

In the primary afferent, transient outward potassium current (I_A) is crucial in controlling electrical excitability (Vydyanathan et al., 2005). Voltage-gated potassium (K_v) channel K_v4 subunits ($K_v4.1$, $K_v4.2$, and $K_v4.3$) and $K_v1.4$ mediate the I_A (Vydyanathan et al., 2005; Phuket and Covarrubias, 2009). On the other hand, peripheral nerve injury is known to reduce the I_A (Everill and Kocsis, 1999) and the expression of dominant isoform $K_v4.3$ located at nonpeptidergic C-fibers over a long period, thereby causing neuronal hyperexcitability underlying neuropathic pain (Kim et al., 2002; Chien et al., 2007). However, the underlying transcriptional mechanisms remain unknown.

Transcription factor-mediated epigenetic mechanisms, such as DNA methylation and histone modifications, regulate the long-lasting transcription, thereby contributing to neuronal functions (Borrelli et al., 2008). Neuron-restrictive silencer factor (NRSF, also known as REST) functions as a repressor of genes, which contain neuron-restrictive silencer element (NRSE, also called RE1). Upon binding to NRSE, NRSF recruits histone deacetylase (HDAC) for generating a repressive chromatin environment (Ballas and Mandel, 2005). Since the presence of NRSE in $K_v4.3$ gene is reported (Otto et al., 2007), we hypothesized that injury causes NRSF-directed epigenetic silencing of $K_v4.3$ gene in the dorsal root ganglion (DRG).

EXPERIMENTAL PROCEDURES

Animals and surgery

Male C57BL/6J mice weighing 20–25 g were used. They were kept in a room with a temperature of 21±2 °C with free access to standard laboratory diet and tap water. The experiments were designed to minimize the number of animals used and their suffering. All procedures were approved by the Nagasaki University Animal Care Committee and were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Partial sciatic nerve ligation was performed as described previously (Inoue et al., 2004).

Oligonucleotide treatments

The antisense oligodeoxynucleotide (AS-ODN) against NRSF (5′-CGGAAGGGCTTGGCC-3′) and its mismatch scrambled oligodeoxynucleotide (MS-ODN; 5′-GTCGTCGGCGGAGCA-3′) were synthesized. AS-ODN and MS-ODN were freshly dissolved in artificial cerebrospinal fluid (aCSF; 125 mM NaCl, 3.8 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, pH 7.4). AS-ODN or MS-ODN was intrathecally injected in a dose of 10 μg/5 μl of aCSF on the first, third, and fifth days, then injury was done with subsequent injections of AS-ODN on days 1, 3, 5, and 6 after injury. The intrathecal injection was administered into the space between the spinal L5 and L6 segments according to the method of Hylden and Wilcox (Hylden and Wilcox, 1980). The messenger RNA (mRNA) expression was assessed at day 7 post-injury.

Quantitative real-time polymerase chain reaction (PCR)

The extraction of total RNA from the ipsilateral L4-6 DRGs and quantitative real-time PCR were performed as described previously (Uchida et al., 2009). The PCR primers used were as follows: for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TATGACTCCACTCACGGCAAAT-3′ (forward) and 5′-GGGTCTCGCTCCTGGAAGAT-3′ (reverse); for $K_v4.3$, 5′-TGCATCTTTCTGGTACACCATAGT-3′ (forward) and 5′-GCTAAAGTTGGAGACTATCACAGG-3′ (reverse). GAPDH was used as an internal control for

[1] These authors have contributed equally to this manuscript.

*Corresponding author. Tel: +81-95-819-2421; fax: +81-95-819-2420.
E-mail address: ueda@nagasaki-u.ac.jp (H. Ueda).

Abbreviations: aCSF, artificial cerebrospinal fluid; AS-ODN, antisense oligodeoxynucleotide; ChIP, chromatin immunoprecipitation; DRG, dorsal root ganglion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacetylase; I_A, transient outward potassium current; K_v, voltage-gated potassium; mRNA, messenger RNA; MS-ODN, mismatch scrambled oligodeoxynucleotide; NRSE, neuron-restrictive silencer element; NRSF, neuron-restrictive silencer factor; PCR, polymerase chain reaction.

0306-4522/10 $ - see front matter © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2009.12.021

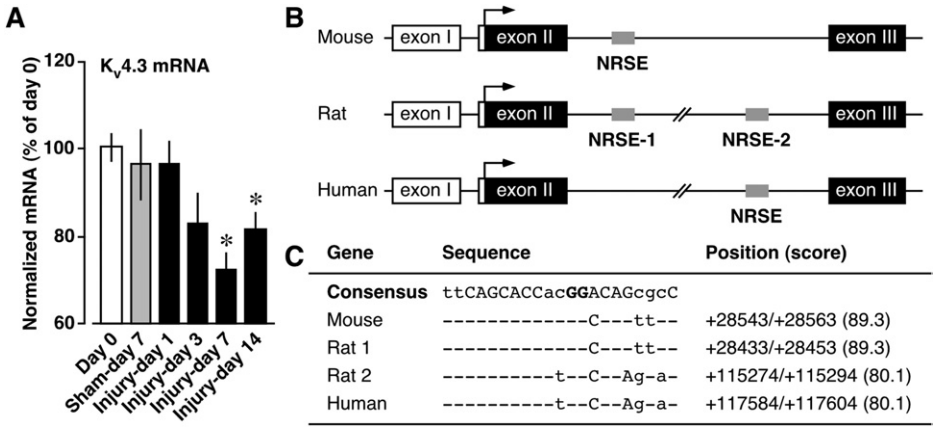

Fig. 1. Downregulation of neuron-restrictive silencer element (NRSE)-containing K_v4.3 gene. (A) Time course of K_v4.3 messenger RNA (mRNA) expressions after injury. Data are calculated as percentages of day 0, and expressed as the means±SEM from at least four mice. * $P<0.05$, vs. day 0. (B) Schematic diagram of locations of K_v4.3-NRSE. The black arrow indicates translation initiation site. The coding exon is shown as black box, the non-coding exon as open box and NRSE sequence as gray box. (C) Deviations of K_v4.3-NRSEs from the consensus NRSE. The capital letters are conserved among functional NRSE sequences, and the bold capital letters are important for neuron-restrictive silencer factor (NRSF) binding. The scores are the threshold scores from the TFSEARCH program.

normalization. In all cases, the validity of amplification was confirmed by the presence of a single peak in the melting temperature analysis and linear amplification with increasing number of PCR cycles. Detailed information of PCR primers was shown in Supplemental Table S1.

Western blot

The ipsilateral L4-6 DRGs were homogenized in ice cold cell-lysis buffer (10 mM Tris–HCl, pH 8.0, 10 mM NaCl, 0.2% NP-40, 1 μM p-APMSF) twice, and then the homogenates were centrifuged to remove contaminating cytosol. Crude nuclear fraction (30 μg) was separated by on 7.5% (NRSF) or 15% (histone H3) SDS-polyacrylamide gels. The primary antibodies were used in the following dilutions: NRSF (1:500; Upstate, CA, USA) and histone H3 (1:500; Upstate, NY, USA). Immunoreactive bands were detected using enhanced chemiluminescent substrate (SuperSignal West Pico chemiluminescent Substrate; Pierce Chemical, Rockford, IL, USA).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed by using protocols from Upstate Biotechnology and a previous report (Kubat et al., 2004) with some modifications. Briefly, chromatin extracted from L4-6 DRGs were cross-linked by 1% formaldehyde, and then sheared by sonication to 200–500 bp fragments. Ten percent of each lysate was used as the input control for normalization. Chromatin was immunoprecipitated with anti-NRSF (5 μg), anti-acetyl-H3 (5 μg; Upstate, Lake Placid, NY, USA) or anti-acetyl-H4 (5 μl antiserum; Upstate, Lake Placid, NY, USA) antibodies, or normal rabbit IgG (5 μg; Santa Cruz, CA, USA). Purified DNA was used for PCR analysis with primers for K_v4.3-NRSE (forward, 5′-ACAACTTAGTTTTGCGCACCAT-3′; reverse, 5′-GCGTGGAACACCTCAAATGT-3′) to amplify the region (35002–35145) of K_v4.3 gene (NC_000069.5). After 38 cycles of amplification (94 °C for 15 s, 59 °C for 30 s, 72 °C for 30 s), 144 bp of PCR products were analyzed on a 2% agarose gel. Quantitative real-time PCR was performed as described above.

Statistical analysis

In Fig. 3, the differences between multiple groups were analyzed using a one-way ANOVA with Tukey–Kramer multiple comparison post-hoc analysis. In Figs. 1A and 2D, mRNA and ChIP data were analyzed using the Student's *t*-test. The criterion of significance was set at $P<0.05$. All results are expressed as means±SEM.

RESULTS

To examine whether K_v4.3 is downregulated at the transcriptional level after injury, we quantified its mRNA expressions in the DRG by real-time PCR. There was a significant reduction in K_v4.3 expression starting from day 7 post-injury, which persisted at least 14 days (Fig. 1A). Using TFSEARCH program (version 1.3, available at: http://www.cbrc.jp/research/db/TFSEARCHJ.html), we found that mouse K_v4.3 gene contains a putative NRSE sequence within intron 2, which is completely conserved in rat (Fig. 1B, C). In addition, another putative conserved NRSE was found in intron 2 of rat and human K_v4.3 genes (Fig. 1B, C). Within all NRSE sequences of these genes, the GG nucleotides known to be important for NRSF binding (Mori et al., 1992), were completely conserved (Fig. 1C).

Using ChIP analysis (Fig. 2A), we found that injury causes a drastic increase in NRSF binding to K_v4.3-NRSE at day 7 post-injury (Fig. 2B), suggesting that this putative NRSE is capable of serving as NRSF-binding site. In the quantitative real-time PCR analysis, the fold-change was not calculated in the NRSF binding to K_v4.3-NRSE, since no significant signal was detected in sham-operated preparations (Fig. 2C). In contrast, negligible binding was observed when precipitated by normal IgG, confirming the specificity of the immunoprecipitation (Fig. 2B, C). Next, we assessed the acetylation of histone H3 and H4, which is correlated with transcriptional activation. Injury caused significant reduction in acetylation of histone H4, but not H3, at K_v4.3-NRSE at day 7 post-injury (Fig. 2D). These data suggest that injury induces repressive chromatin states around K_v4.3-NRSE possibly through NRSF-HDAC-mediated mechanisms.

For selective knockdown of NRSF expression, AS-ODN designed to target the mouse NRSF sequence,