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TUMOR NECROSIS FACTOR-ALPHA INDUCES EXPRESSION OF C/EBP-BETA IN PRIMARY AFFERENT NEURONS FOLLOWING NERVE INJURY

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Abstract—CCAAT/enhancer binding protein-beta (C/EBP-beta) is a transcription factor that belongs to the C/EBP family. To understand the role of C/EBP-beta in the peripheral nervous system, we investigated the expression of C/EBP-beta in the dorsal root ganglion. C/EBP-beta was weakly detected in nuclei of naive dorsal root ganglion (DRG) neurons. Spinal nerve ligation increased the expression of C/EBP-beta in L4 and L5 DRG neurons. Treatment with anti-TNF-alpha prevented SNL-induced pain hypersensitivity and C/EBP-beta expression in the DRG. Injection of TNF-alpha into the sciatic nerve produced transient pain hypersensitivity and induction of C/EBP-beta expression in the DRG. These results demonstrate that C/EBP-beta is activated in the DRG neurons by a TNF-alpha-dependent manner and might be involved in the activation of primary afferent neurons after nerve injury.

Key words: C/EBP-beta, nerve injury, dorsal root ganglion, TNF-alpha.

INTRODUCTION

Injury or inflammation of peripheral nerves results in neuropathic pain, which is characterized by allodynia and hyperalgesia. After peripheral nerve injury, increased electrical activity occurs in the injured primary sensory neurons as well as in adjacent intact afferents (Campbell, 2001). Altered neuronal excitability after peripheral nerve injury is caused by modulated gene expression, leading to changes associated with pain perception, transduction and translation (Campbell and Meyer, 2006). However, intracellular mechanisms regulating gene expression in primary afferent neurons following nerve injury are not known.

The CCAAT/enhancer binding protein (C/EBP) family is composed of basic leucine zipper transcription factors that can recognize a common cis-regulatory DNA element (Ramji and Foka, 2002; Schrem et al., 2004). C/EBPs are present in various tissues, including the brain (Sterneck and Johnson, 1998; Sterneck et al., 1998; Nadeau et al., 2005). C/EBP-beta (also known as NF-IL6) is one of the C/EBP families identified as a regulator of gene transcription in response to IL-1 and IL-6 (Akira et al., 1990; Poli et al., 1990). Subsequently, C/EBP-beta was shown to be involved in various brain functions, such as synaptic plasticity associated with learning and memory (Taubenfeld et al., 2001).

Less is known about the role of C/EBP-beta in the peripheral nervous system. In the present study, we investigated expression, and its regulation, of C/EBP-beta in primary afferent neurons after nerve injury. We found that C/EBP-beta was expressed in these primary afferent neurons, and was increased in animal models for neuropathic pain via TNF-alpha-dependent manner, suggesting that it plays an important role for the nerve injury-induced activation of primary afferent neurons.

EXPERIMENTAL PROCEDURES

Animals and surgery

The Kyoto Prefectural University of the Medicine Animal Care Committee approved all experimental procedures, which were implemented according to the guidelines of the National Institutes of Health and the International Association for the study of Pain (Zimmermann, 1983). Male Sprague–Dawley rats (Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan) weighing 200–250 g were housed in groups of three per cage on a 12-h light/dark cycle. The rats were anesthetized with isoflurane (2%).

Spinal nerve ligation (SNL). To produce the SNL model, the L6 transverse process was removed to expose the L4 and L5 spinal nerves. The L5 spinal nerve was isolated carefully and ligated tightly with 3–0 silk thread (Kim and Chung, 1992). To see the effect of TNF-alpha inhibitor on pain hypersensitivity and C/EBP-beta expression after the SNL, TNF-alpha inhibitor WP9QY (1 mg/ml, 500 μl per animal); Merck Chemicals, Darmstadt, Germany, dissolved in 20% DMSO) (Takasaki et al., 1997) was systemically administered via an intraperitoneal injection 30 min before SNL. Control

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Abbreviations: ANOVA, analysis of variance; ATF, activating transcriptional factor; C/EBP-beta, CCAAT/enhancer binding protein-beta; DRG, dorsal root ganglion; SNL, spinal nerve ligation; TSA, Tyramide Signal Amplification.

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animals received 20% DMSO intraperitoneally then performed SNL surgery.

TNF-alpha treatment. To investigate the effect of TNF-alpha on the expression of C/EBP-beta, recombinant rat TNF-alpha (10 pg/ml, 10 μl per animal); R&D Systems, Inc., Minneapolis, MN USA, dissolved in 0.1 M PBS) was administered into the left perisciatic nerve after aseptic exposure of the sciatic nerve at mid-thigh level by blunt dissection as described previously (Shibasaki et al., 2010). Control animals received 0.1 M PBS. Twelve hours after the treatment with TNF-alpha, animals were perfused and L4 and L5 dorsal root ganglion (DRG) were taken to process immunohistochemistry.

Behavioral assessments

All experimental tests were performed at the same time of day and by an experimenter unaware of the treatment applied. Nociceptive hind paw threshold to mechanical and thermal stimulus was tested in 8 animals. Rats were habituated to the experimental room for 30 min. All animals were placed in a clear plastic chamber on an elevated wire grid. For mechanical paw-withdrawal threshold, the plantar surface of the hindpaw was stimulated with a series of von Frey hairs. Withdrawal responses to mechanical stimulation were determined using a calibrated von Frey monofilament set (Stoelting Company, Wood Dale, IL, USA). The threshold was taken as the lowest force that evoked a clear withdrawal response at least twice in 10 applications. To measure thermal sensitivity, animals were placed on a hot plate (IITC Life Science Instruments Model 39D; Woodland Hills, CA, USA) maintained at 50 °C. Time until licking or biting left hindpaw or jumping was recorded as latency time. A cut-off time of 25 s was set to prevent skin injury. Score of heat hypersensitivity was averaged for three measurements of latency for each rat in each test session. The mean of three measurements taken between 10 and 15 min apart was taken as the latency to paw withdrawal.

Tissue preparation and immunohistochemistry

Expression of C/EBP-beta in the DRG was examined in animals treated with SNL (1–7 days after the surgery) and peri-sciatic administration of TNF-alpha. Animals were perfused with 250 ml of 0.9% NaCl followed by 300 ml of 10% neutralized formalin (Wako, Osaka, Japan) under terminal anesthesia. The L4 and L5 DRG were removed and post fixed for 1 h in the same fixative solution, then cryoprotected in 20% sucrose in 0.1 M PB for 24 h at 4 °C. Tissues were then stored frozen at -80 °C. DRG sections (10 μm) were cut using by a cryostat (Leica Biosystems, Nussloch, Germany) and mounted on Silane-coated glass slides.

Sections were washed twice with 0.1 M PBS, incubated with rabbit anti- C/EBP-beta antibody (1:400, Active Motif, CA, USA) in 0.1% Tween 20 in TNB buffer (0.1 M Tris buffered saline, pH 7.4, containing 1% blocking reagent) for 48 h at 4 °C. The sections were washed twice with 0.1 M PBS and incubated with Rhodamine-conjugated anti-rabbit antibody (1:1000, Merck Millipore, Billerica, MA, USA) overnight at 4 °C. For double staining immunohistochemistry, sections from L4 DRG taken 3 days after the SNL were incubated with rabbit anti-C/EBP-beta antibody (1:400), mouse anti-NF200 antibody (marker for myelinated DRG neuron) (1:2000, Sigma–Aldrich, St. Louis, MO, USA) or biotin-conjugated isolectin B4 (IB4) from griffonia simplicifolia (10 μg/ml, Sigma–Aldrich) in 0.1% Tween20 in TNB for 48hr at 4 °C. The sections were washed twice with 0.1 M PBS and incubated with Rhodamine-conjugated anti-rabbit antibody (1:1000), FITC-conjugated anti-mouse antibody (1:2000, Merck Millipore) or FITC-conjugated anti-biotin antibody (1:1000, Vector laboratories, Burlingame, CA) for 48 h at 4 °C. Double staining immunohistochemistry for C/EBP-beta and ATF-3 was performed by the Tyramide Signal Amplification (TSA; Perkin Elmer, Waltham, MA, USA) system (Amaya et al., 2000). Following primary antibody incubation with rabbit anti-C/EBP-beta antibody (1:10,000) in TNB buffer for 48hr at 4 °C, signals were visualized by the TSA indirect method with streptavidin-Alexa Fluor 594. Sections were then incubated with rabbit anti-ATF-3 antibody (1:1000; Santa Cruz Biotechnology Dallas, Texas) in TNB for 48hr at 4 °C, followed by incubation with Rhodamine-conjugated Dky-anti-Rabbit IgG antibody (1:1000; Merck Millipore) in TNB for 24 h at 4 °C. The sections were washed and visualized using a fluorescence microscope with a digital camera system (Nikon, Tokyo, Japan).

Cell count

Four tissue sections (minimum separation of 100 μm) were randomly selected from one DRG to analyze the proportion of C/EBP-beta-positive neurons. The ratio of immuno-positive nucleated cells compared to the total number of nucleated cells was calculated in a blinded manner to identify C/EBP-beta NF200, IB4 or ATF3 expression in the DRG.

Western blotting

Recombinant human C/EBP-beta protein (0.1 μg, Abnova, Taipei, Taiwan) was loaded on sodium dodecyl sulfate polyacrylamide gel and transferred to a PVDF membrane. The membrane was incubated with rabbit anti-C/EBP-beta antibody (1:1000). The signal was developed with the ECL Western blotting Analysis System (GE Healthcare Bioscience, Piscataway, NJ, USA), and visualized with the EZ-Capture II CCD Camera System (ATTO Corporation, Tokyo, Japan). MagicMark XP Western Standard (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA) was used for the estimation of the molecular weight.

Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's post-test or a two way ANOVA with Bonferroni's post-test was performed using GraphPad Prism5 (GraphPad Software, La Jolla, CA, USA) on a Macintosh computer system. Values of $P < 0.05$ were considered to be statistically significant.