Activation of p38 Mitogen-Activated Protein Kinase in Spinal Hyperactive Microglia Contributes to Pain Hypersensitivity Following Peripheral Nerve Injury

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ABSTRACT Neuropathic pain is an expression of pathological operation of the nervous system, which commonly results from nerve injury and is characterized by pain hypersensitivity to innocuous stimuli, a phenomenon known as tactile alldynia. The mechanisms by which nerve injury creates tactile alldynia have remained largely unknown. We report that the development of tactile alldynia following nerve injury requires activation of p38 mitogen-activated protein kinase (p38MAPK), a member of the MAPK family, in spinal microglia. We found that immunofluorescence and protein levels of the dually phosphorylated active form of p38MAPK (phospho-p38MAPK) were increased in the dorsal horn ipsilateral to spinal nerve injury. Interestingly, the phospho-p38MAPK immunofluorescence in the dorsal horn was found exclusively in microglia, but not in neurons or astrocytes. The level of phospho-p38MAPK immunofluorescence in individual microglial cells was much higher in the hyperactive phenotype in the ipsilateral dorsal horn than the resting one in the contralateral side. Intrathecal administration of the p38MAPK inhibitor, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), suppresses development of the nerve injury-induced tactile alldynia. Taken together, our results demonstrate that nerve injury-induced pain hypersensitivity depends on activation of the p38MAPK signaling pathway in hyperactive microglia in the dorsal horn following peripheral nerve injury. © 2003 Wiley-Liss, Inc.

INTRODUCTION

Nerve injury arising from disease or physical trauma produces long-lasting abnormal hypersensitivity to innocuous stimuli, a phenomenon known as tactile alldynia (Woolf and Mannion, 1999; Scholz and Woolf, 2002). Tactile alldynia is a hallmark, and the most troublesome, of neuropathic pain syndrome in humans. It is nearly always resistant to known treatments such as nonsteroidal antiinflammatory agents (NSAIDs) or even narcotics (Woolf and Mannion, 1999; Scholz and Woolf, 2002). The mechanisms by which nerve injury develops tactile alldynia have remained largely unknown. It is thus essential to identify the molecular changes that lead to tactile alldynia in an effort to both understand its mechanisms and develop new therapies.

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Several lines of evidence have proposed that induction of tactile allodynia is attributed to central hyperactive states resulting from multiple plastic alterations in dorsal horn neurons as well as glia following nerve injury (Woolf and Mannion, 1999; Woolf and Salter, 2000; Watkins et al., 2001; Scholz and Woolf, 2002). Recent models of nerve injury-induced plasticity in the dorsal horn postulate that induction of the spinal plasticity requires activation of intracellular signaling events including protein kinases for transcriptional and posttranscriptional modifications of various proteins such as cell surface receptors (Woolf and Salter, 2000). It is thus expected that protein kinases, including protein kinase C (Woolf and Salter, 2000), protein kinase (p38MAPK), one of four subgroups of the MAPK family (Ono and Han, 2000); activation of p38MAPK is found in hyperactive microglia, but not in neurons or astrocytes in the dorsal horn after nerve injury. Thus, the present study suggests that p38MAPK in spinal microglia is an essential intracellular protein kinase that regulates pain hypersensitivity following peripheral nerve injury. Preliminary results of this study have been reported in abstract form (Tsuda et al., 2002a,b).

MATERIALS AND METHODS

Animals

Male Wistar rats were used in this study: rats weighing 200–230 g for the biochemical and immunohistochemical experiments, and rats weighing 270–290 g for the behavioral experiments testing the effect of intrathecal treatment with a p38MAPK inhibitor. We have confirmed that p38MAPK phosphorylation in the dorsal horn following nerve injury is also observed in both weight ranges of rats (data not illustrated). Rats were housed at a temperature of 22 ± 1°C with a 12-h light/dark cycle (light on 8:30 to 20:30) and were fed food and water ad libitum. All the animals used in the present study have been treated in accordance with the guidelines of National Institute of Health Sciences.

Neuropathic Pain Model

We used the spinal nerve injury model (Kim and Chung, 1992) with some modifications. A unilateral L5 spinal nerve of rats was tightly ligated and cut just distal to the ligature under isoflurane (2%) anesthesia. To assess the tactile allodynia, the calibrated von Frey filaments (0.4–15.1 g; Stoelting, Wood Dale, IL) were applied to the plantar surface of the hindpaw from below the mesh floor. The 50% paw withdrawal threshold was determined by the up-down method (Dixon, 1980; Chaplan et al., 1994).

Immunohistochemistry

The rats were deeply anesthetized by pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 150 ml of phosphate-buffered saline (PBS; composition in mM: NaCl 137, KCl 2.7, KH2PO4 1.5, NaH2PO4 8.1; pH 7.4), followed by 300 ml of ice-cold 4% paraformaldehyde. The L5 segment of the lumbar spinal cord was removed, postfixed in the same fixative, and placed in 30% sucrose solution for 24 h at 4°C. Transverse L5 spinal cord sections (30 μm) were incubated in a blocking solution (3% normal goat serum [NGS]) and then incubated for 48 h at 4°C in the primary antibody, anti-phospho-p38MAPK (1:200; Cell Signaling, Beverly, MA). Markers of microglia, OX42 (anti-OX42, 1:100, Chemicon, Temecula, CA); astrocytes, glial fibrillary acidic protein (GFAP; anti-GFAP, 1:500; Boehringer-Mannheim, Indianapolis, IN); and neurons, NeuN (anti-NeuN, 1:200; Chemicon) were used to identify the type of phospho-p38MAPK-positive cells. Following incubation, tissue sections were washed and incubated for 3 h at room temperature in the secondary antibody solution (anti-rabbit IgG-conjugated Alexa Fluor™ 488 or anti-mouse IgG-conjugated Alexa Fluor 546, 1:1,000; Molecular Probes, Eugene, OR). The spinal cord sections were analyzed using a MicroRadiance Confocal Imaging System (Bio-Rad, Hercules, CA) and an Olympus IX70 microscope (Olympus Optical, Tokyo, Japan) equipped for epifluorescence. For quantitative assessment of the immunofluorescence staining of cells, we randomly selected dorsal horn fields displayed at high magnification. Microglia, as identified by OX42 immunofluorescence, were outlined; the immunofluorescence intensity of the phospho-p38MAPK was determined as the average pixel intensity within each cell. Background fluorescence intensity was determined and was subtracted from the value obtained for microglia.

Western Blotting

The rats were deeply anesthetized with pentobarbital (100 mg/kg, i.p.). The lumbar and sacral spinal cord was quickly removed and placed on a dish with ice-cold PBS. We identified the cord from L4 to L6 by the entry area of the dorsal roots and the shape of the cord under a microscope and cut at the boundary between L3 and L4 and between L6 and S1. The spinal cord segments L4-L6 ipsilateral to the nerve injury were homogenized in ice-cold PBS containing a mixture of phosphatase inhibitors (Sigma-RBI, St. Louis, MO) and protease inhibitors (Calbiochem, San Diego, CA). The homogenates were incubated with DNase and were sonicated. The resulting homogenate (20 μg) was subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred electrophoretically to nitrocellulose membranes. After blocking, the membranes were incubated with anti-phospho-p38MAPK antibody (1:1,000; Cell Signal-
ing) or anti-p38MAPK antibody (1:1,000; Cell Signaling) and then were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. The blots were detected with a chemiluminescence method (LumiGLO; Cell Signaling) and exposed to autoradiography films (Hyperfilm-ECL; Amersham, Arlington Heights, IL).

**Spinal Administration of p38MAPK Inhibitor**

Under isoflurane (2%) anesthesia, rats were implanted with catheters for intrathecal injection according to the method described previously (Yaksh et al., 1980). A polyethylene tube (PE-10; 7.5 cm) was inserted through the atlanto-occipital membrane and to the lumbar enlargement (close to L4-L5 segments) and externalized through the skin. Rats were injected intrathecally with 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580; 30 nmol/10 μl) (Sigma-RBI) or vehicle [2% dimethylsulfoxide (DMSO)/10 μl] using a 25-μl Hamilton syringe with 28-gauge needle. Intrathecal injection of SB203580 or vehicle was started immediately after nerve injury (day 0) and given once a day for 14 days. SB203580 was dissolved in 100% DMSO and diluted by PBS (final concentration of DMSO: 2%). Behavioral testing was done 12–14 h after the injection of SB203580.

**Statistical Analysis**

Statistical analyses of the results were evaluated using the Student’s t-test, the Student’s paired t-test or the Mann-Whitney U-test.

**RESULTS**

**p38MAPK Is Activated Exclusively in Hyperactive Microglia in the Dorsal Horn Following Peripheral Nerve Injury**

Animals with L5 spinal nerve injury displayed tactile allodynia; the withdrawal threshold of the hindpaw, ipsilateral to nerve injury, to mechanical stimulation decreased progressively from 15.1 ± 0.1 g before the injury (day 0) to 3.0 ± 0.5 g at day 7 and to 2.1 ± 0.4 g (n = 7) at day 14 (P < 0.001, significantly different from the threshold on day 0) (Fig. 1A). In contrast, paw withdrawal threshold of the contralateral hindpaw was not changed significantly by nerve injury (Fig. 1A). To examine whether p38MAPK is activated in the dorsal horn of the spinal cord in rats that have developed tactile allodynia, we carried out immunofluorescence analysis with an antibody targeting the dually phosphorylated p38MAPK (phospho-p38MAPK), because p38MAPK members have a Thr-Gly-Tyr dual phosphorylation motif, requiring phosphorylation for its activation (Ono and Han, 2000). In L5 dorsal spinal cord sections, at 14 days after nerve injury, we observed strong and punctate phospho-p38MAPK immunofluorescence on the ipsilateral side (Fig. 1B). The punctate labeling observed at low magnification was due to immunofluorescence of individual small cells, as shown under highly magnification (Fig. 1B, inset). In contrast, phospho-p38MAPK immunofluorescence was weaker and much less extensive in the dorsal horn control.
Fig. 2. p38 mitogen-activated protein kinase (p38MAPK) is activated in individual hyperactive microglia, but not in neurons or astrocytes in the dorsal horn following L5 spinal nerve injury. Immunostaining was carried out in L5 dorsal spinal cord sections at 14 days after nerve injury, using confocal microscopy. A–C: Double immunofluorescent labels of phospho-p38MAPK (green) with NeuN (A, red), a marker of neurons, glial fibrillary acidic protein (GFAP) (B, red), a marker of astrocytes, and OX42 (C, red), a marker of microglia, were analyzed. D: The change of the level of OX42 immunofluorescence (red) following nerve injury was examined in transverse section of L5 dorsal horn at 14 days after nerve injury. E: The different activation of p38MAPK in ipsilateral (Ipsi, top three panels) and contralateral (Contra, bottom three panels) microglia was examined. OX42, phospho-p38MAPK (p-p38) and merged immunofluorescences are shown in red, green, and yellow, respectively. F: Immunofluorescence intensity of phospho-p38MAPK in individual microglia was determined as the average pixel density in the ipsilateral (Ipsi; n = 83 OX42-positive cells) and contralateral (Contra; n = 74 OX42-positive cells) dorsal horn. Each data point represents the mean ±SEM of immunofluorescence (IF) intensity of phospho-p38MAPK (p-p38) per cell (***P < 0.001 by the Mann-Whitney U-test, compared with the value of contralateral dorsal horn). G: Histogram of the percentage of dorsal horn microglia displaying ranges of IF intensity values of phospho-p38MAPK in individual microglia. Ipsi, ipsilateral; Contra, contralateral. Scale bars = 25 μm in C; 200 μm in D; 10 μm in E.

To identify the type of cell in which p38MAPK was phosphorylated after nerve injury, we carried out double immunolabeling for phospho-p38MAPK and for cell type-specific markers: for neurons, NeuN; for astrocytes, GFAP; or for microglia, OX42 (Honore et al., 2000). We found that cells showing phospho-p38MAPK immunofluorescence were not double-labeled for NeuN (0%, calculated in 110 cells, representative shown in Fig. 2A) or GFAP (0%, calculated in 132 cells, representative shown in Fig. 2B). Rather, almost all phospho-p38MAPK-positive cells (99%, calculated in 187 cells) were double-labeled with OX42 (representative shown in Fig. 2C), indicating that activation of p38MAPK in the dorsal horn is highly restricted to neuronal and astrocytic cells.
microglia, but not found in neurons or astrocytes. OX42 recognizes the complement receptor type 3 (CR3), expression of which is greatly increased in hyperactive versus resting microglia (Aldskogius and Kozlova, 1998). We found that OX42 labeling was greater in the dorsal horn ipsilateral to the nerve injury (Fig. 2D), whereas OX42 labeling in the dorsal horn was low bilaterally in sham-operated animals (not illustrated). OX42-positive cells were more numerous (Fig. 2D) and displayed hypertrophic morphology (Fig. 2E) in the dorsal horn on the side of the nerve injury as compared with the contralateral side (Fig. 2D,E). These results indicate that nerve injury induced a switch from the resting to the hyperactive phenotype in the population of microglia in the dorsal horn. The cells labeled intensely with OX42 showed high levels of phospho-p38MAPK immunofluorescence (Fig. 2E, top panels). In contrast, resting microglia that showed a low level of OX42 had no or weak phospho-p38MAPK immunofluorescence (Fig. 2E, bottom panels). The mean level of intensity of phospho-p38MAPK immunofluorescence per OX42-positive cell was on average 3.7-fold higher in the ipsilateral (n = 83 cells) as compared with the contralateral dorsal horn (n = 74 cells) (P < 0.001; Fig. 2F). The distribution of phospho-p38MAPK immunofluorescence intensities per OX42-positive cell was skewed to the right (Fig. 2G). We conclude that, in the dorsal horn following nerve injury, hyperactive microglia are the cell type in which p38MAPK is activated and that the level of p38MAPK phosphorylation is dramatically increased in individual microglia. As shown in Figure 2E, subcellular distribution between phospho-p38MAPK and OX42 immunofluorescence is different, but our confocal microscopic Z-series analyses demonstrated that phospho-p38MAPK signals were found in the inside of OX42 signals which is known to localize on the cell surface (data not illustrated).

**p38MAPK Activation in the Spinal Cord Is Required for Development of Tactile Allodynia Following Peripheral Nerve Injury**

We examined whether intrathecal treatment with a potent inhibitor of p38MAPK, SB203580, through a catheter whose tip was positioned near the L4-L5 dorsal horn alters the development of tactile allodynia following nerve injury. Catheterized rats were treated with vehicle (2% DMSO/10 μl, n = 7) or SB203580 (30 nmol/10 μl, n = 9) once a day for 14 days, beginning on the day of the nerve injury. Intrathecal vehicle-treated rats displayed a marked decrease in paw withdrawal threshold following nerve injury (P < 0.01, significantly different from the threshold on day 0) (Fig. 3). In contrast, intrathecal SB203580-treated rats showed only a slight decrease in paw withdrawal threshold; paw withdrawal thresholds was not significantly decreased except for day 3 (P < 0.01, significantly different from the threshold on day 0). Paw withdrawal thresholds on day 7 and 14 were significantly greater in animals treated with SB203580 (n = 9) as compared with that in animals treated with vehicle (n = 7) (day 7: P < 0.01, day 14: P < 0.001, significantly different from the threshold of vehicle-treated group on days 7 and 14, respectively) (Fig. 3). These results suggest that intrathecal treatment with an inhibitor for p38MAPK in the spinal cord, the distribution of which is highly restricted in microglia, suppresses the development of tactile allodynia following spinal nerve injury.

**DISCUSSION**

Our principal conclusion from the present findings is that following spinal nerve injury p38MAPK is activated in individual hyperactive microglia in the dorsal horn, leading to the development of pain hypersensitivity tactile allodynia, a major functional consequence of peripheral nerve injury. p38MAPK is the first intracellular signaling event, activation of which occurs exclusively in microglia, that regulates pain hypersensitivity caused by nerve injury. We showed a marked increase in immunofluorescence and protein levels of dual-phosphorylated p38MAPK in the dorsal horn after spinal nerve injury. These results are supported by previous findings that phosphorylation of p38MAPK is increased in response to the damage of the sciatic nerve or dorsal root (Murashov et al., 2001; Nomura et al., 2001; Kim et al., 2002) that projects to the dorsal spinal cord. p38MAPK has been
reported to be expressed in a number of cell types in the CNS in vivo (Lee et al., 2000; Maruyama et al., 2000). We found that in the neuropathic pain state, p38MAPK activation in the dorsal horn was not observed in neurons or astrocytes but, rather, occurred exclusively in microglia. As shown previously (Aldskogius and Kozlova, 1998), the number of microglia increased in the dorsal horn on the side of the nerve injury following nerve injury. We have recently demonstrated that the number of microglia is on average 2.2-fold greater in the ipsilateral side of the dorsal horn than in the contralateral side (Tsuda et al., 2003). We also show that marked phosphorylation of p38MAPK is observed in individual microglia in the ipsilateral dorsal horn (3.7-fold, as compared with the contralateral side), particularly in hyperactive microglia that dramatically expressed complement receptor type 3 recognized by OX42, and displayed hypertrophic morphology. The increase is more striking than the increase in the number of microglia in the ipsilateral side of the dorsal horn. Therefore, in the dorsal horn, following nerve injury, hyperactive microglia are the cell type in which p38MAPK is activated; in addition to an increase in the number of cells, the increased p38MAPK phosphorylation in individual hyperactive microglia would be one of the major components of p38MAPK activation in the dorsal horn following nerve injury. Moreover, we show that intrathecal treatment with SB203580, which binds to the ATP pocket in p38MAPK, and consequently inhibits its enzymatic activity (Tong et al., 1997), led to a statistically significant suppression of tactile allodynia on days 7 and 14 after nerve injury when the increase in p38MAPK phosphorylation was found. It appears that the suppression of tactile allodynia by SB203580 might be related to its inhibitory effect on the increased p38MAPK activity in the dorsal horn after nerve injury, based on the observations that intrathecal administration of SB203580 has no effect on basal pain responses in naive rats, which have a very low level of p38MAPK phosphorylation (Watkins et al., 1997; Murashov et al., 2001; Nomura et al., 2001; Ji et al., 2002; present data). Taking these results together, we conclude that development of tactile allodynia following nerve injury depends on activation of the p38MAPK signaling pathway in hyperactive microglia in the dorsal horn, although we cannot exclude the possible involvement of p38MAPK in DRG neurons (Kim et al., 2002).

p38MAPK activation in microglia is quite different from that of other MAPKs, extracellular signal-regulated kinases (ERK) and c-jun N-terminal or stress-activated protein kinases (JNK/SPAK), activation of which is found in dorsal horn astrocytes, but not in microglia, after peripheral nerve injury (Ma and Quirion, 2002). Thus, microglial regulation of tactile allodynia may require activation of p38MAPK, but not other MAPKs. Several extracellular substances have been reported to trigger p38MAPK activation in microglia in vitro, thereby regulating microglial functions (Koistinaho and Koistinaho, 2002). Tikka et al. (2001) have shown that glutamate-evoked proliferation of microglia and interleukin-1β (IL-1β) and nitric oxide release from microglia in the spinal cord primary culture depend on its p38MAPK activation. We have recently demonstrated that extracellular ATP activates p38MAPK in cultured microglia, thereby releasing tumor necrosis factor-α (TNF-α) and IL-6 (Hide et al., 2000; Shigemoto-Mogami et al., 2001). These cytokines are increased in the spinal cord following spinal nerve injury (Sweitzer et al., 2001; Winkelstein et al., 2001) and are involved in induction of nerve injury-induced tactile allodynia (Ramer et al., 1998; Sommer et al., 1998; Sweitzer et al., 2001). Therefore, elucidating p38MAPK activity-dependent microglial outputs, including the production of these cytokines in the dorsal horn in vivo, would help in understanding the mechanisms underlying the induction of pain hypersensitivity following nerve injury.

Nerve injury and peripheral inflammation that produce neuropathic and inflammatory pain states, respectively, have been known to induce distinct sets of neurochemical changes in the dorsal horn (Honore et al., 2000). In contrast to the present findings with peripheral nerve injury, Ji et al. (2002) have shown that p38MAPK activation does not occur in the dorsal horn under a sustained inflammation by intraplantar injection of complete Freund’s adjuvant, which produces prolonged hypersensitivity to pain. Thus, p38MAPK activation in dorsal horn microglia would be a unique intracellular change following nerve injury, contributing to the development of nerve injury-induced pain hypersensitivity. This approach may provide a new therapeutic strategy specially targeting neuropathic pain. Importantly, in naive animals, p38MAPK activation is very weak in the dorsal horn (Fig. 1), and basal pain sensitivity is not affected by spinal administration of p38MAPK inhibitors (Watkins et al., 1997; Ji et al., 2002). This suggests a therapeutic benefit of interfering with p38MAPK activation in the treatment of neuropathic pain, without affecting normal pain sensitivity.

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