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Microglial activation of p38 contributes to scorpion envenomation-induced hyperalgesia

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ABSTRACT

Intraplantar (i.pl.) injection of BmK I, a receptor site 3-specific modulator of voltage-gated sodium channels (VGSCs) from the venom of scorpion *Buthus martensi* Karsch (BmK), was shown to induce long-lasting and spontaneous nociceptive responses as demonstrated through experiments utilizing primary thermal and mirror-imaged mechanical hypersensitivity with different time course of development in rats. In this study, microglia was activated on both sides of L4–L5 spinal cord by i.pl. injection of BmK I. Meanwhile, the activation of p38/MAPK in L4–L5 spinal cord was found to be co-expressed with OX-42, the cell marker of microglia. The unilateral thermal and bilateral mechanical pain hypersensitivity of rat induced by BmK I was suppressed in a dose-dependent manner following pretreatment with SB203580 (a specific inhibitor of p-p38). Interestingly, microglia activity was also reduced in the presence of SB203580, which suggests that BmK I-induced microglial activation is mediated by p38/MAPK pathway. Combined with previously published literature, the results of this study demonstrate that p38-dependent microglial activation plays a role in scorpion envenomation-induced pain-related behaviors.

1. Introduction

Scorpion *Buthus martensi* Karsch (BmK) envenomation can cause intense pain, skin edema, and burning sensation at the site of the sting [1]. BmK I, a voltage-gated sodium channels (VGSCs) receptor site 3-specific modulator purified from BmK venom, is considered to be the key contributor of scorpion envenomation induced pain-related behaviors [2].

Glial cells provide supportive functions in the central nervous system (CNS), and have been demonstrated to be capable of communicating with neurons to contribute to their normal growth, development, differentiation and maturation. At the same time they have also been implicated to play a role in diseases of the CNS and in the facilitation of pain sensations [3–5]. For instance, activation of spinal non-neuronal cells, such as microglia and astrocytes, have been shown to be involved in the generation and maintenance of enhanced states of pain [6]. Similarly, drugs that act as inhibitors or modifiers of glial activity, such as fluoroacitrate and propentofylline, have been noted to alter abnormal pain sensitivity [7].

The activation of spinal microglia, in both neuropathic and inflammatory animal pain models, is accompanied by changes in morphology (from ramified to amoeboid), gene expression (e.g., MCH I and II, CD11b, Iba1), function (phagocytosis), and/or cell number (proliferation) [8]. As a source of cell factors, activated microglia releases a number of inflammatory and signaling molecules [9]. However, little is known in terms of how microglia is excited in mechanisms involved in pain sensation.

The mitogen-activated protein kinases (MAPKs) – including extracellular signal-regulated kinase 1/2 (ERK1/2), p38, c-Jun N-terminal kinase (JNK), and ERK5 – are a family of serine/threonine protein kinases that have been identified as candidates responsible for downstreaming the G protein-coupled receptors that contribute to the transduction of extracellular stimuli to produce a diverse range of intracellular responses [10]. For instance, the activation of MAPK within the spinal cord has been implicated in a variety of enhanced pain states [11]. The extracellular signals that result from MAPK activation in the spinal cord are integrated and conveyed into the cytoplasm and nucleus by p38 phosphorylation [12]. The mobilization of p38 in spinal glia may contribute to pain development and/or maintenance of pain sensation resulting from inflammation or nerve injury [13,14]. In addition, it has clearly established that p38 MAPK is involved in the production of inflammatory cytokines such as IL-1 and TNF [15].

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However, the link between the p38 pathway and microglial activation in pain responses, particularly induced by scorpion envenomation, has not been discussed in the literature. Thus, the current study seeks to address the knowledge gap by investigating: (1) the association of i.pl. BmK I injection and p38 activity; (2) the cellular distribution of p38 activity post-i.pl. BmK I injection; and (3) the role of p38 activity in sustained mechanical and thermal hypersensitivity following i.pl. administration of BmK I.

2. Materials and methods

2.1. Experimental animals

Male adult Sprague–Dawley rats weighing from 220–250 g were purchased from SLAC Laboratory Animal Center, Shanghai. All experiments followed European Community guidelines for the use of experimental animals and guidelines of International Association for the Study of Pain for pain research [16].

2.2. Preparation and administration of BmK I

BmK I used in this study was purified from the venom of scorpion BmK following the process described by Ji et al. [2], and then assessed by mass spectrum to ensure there is a single peak. Pain sensation was induced by BmK I which was intraplantar (i.pl.) injected into the left rat hind paw [1].

2.3. Preparation and administration of SB203580

The 4-(4-fluorophenyl)-2-(4-methyl-sulfonylphenyl)-5-(4-pyridyl)-1H- imidazole (SB203580), a specific inhibitor of p38 MAPK, has been widely used in the investigation of the role of p38 MAPK in various physiological processes, including inflammatory pain [17]. SB203580 was dissolved in 25% dimethyl sulfoxide (DMSO, v/v in saline). All drugs were purchased from Sigma. Three doses of SB203580 (2, 10, or 50 μg) were each administrated intrathecally (i.t.) 30 min before BmK I i.pl. injection. Rats treated with 25% DMSO were used as control. As described previously, 10 μl of SB203580 or DMSO were directly injected by lumbar puncture at the L5–L6 spinal cord [18].

2.4. Immunohistochemistry

The rat lumbar spinal cord was collected according to previous description. Frozen serial coronal sections of spinal cord (14 μm in thickness) were pasted on to gelatin-coated glass slides and stored under −20 °C. The single immunolabeling used the avidin–biotin–peroxidase complex (ABC) method [19,20]. Nine to twelve sections were randomly selected from each group (3 rats) and averaged. The immunoreactive data (area of immunostaining & No. of positive cells) was analyzed with a computer-assisted image analyzer (Image Pro-plus 6.0, Kodak, USA). The threshold level for immunostaining against background was defined as the mean background gray value in unstained areas plus three standard deviations. Intensities exceeding the threshold were accepted as true immunostaining as shown in supplementary figure (Fig. S1B & C) [21,22]. Quantitative measurements were made in the spinal cord dorsal horn. As measuring areas, three random fields of 500 * 500 pixel in each interested area were defined (Fig. S1A). The average positive area in the three defined fields presents the immunoreaction level.

In the double immunolabeling experiment, the sections were incubated with first primary antibody for 24 h at 4 °C and then incubated with corresponding secondary antibody. After rinsing of PBS, repeated steps described above. The sections were cover slipped at last. Digital images were captured from fluorescent microscopy (Leica, Germany) then merged by Image J software.

Followed antibody products were used in this study: rabbit polyclonal anti-phospho-p38 MAPK primary antibody (1:200; Cell signaling Technology, USA), mouse monoclonal anti-OX-42 (CD11b, microglia marker, 1:50; Santa Cruz Biotechnology), mouse monoclonal anti-glial fibrillary acidic protein (GFAP, astrocyte marker, 1:300; Cell Signaling Technology), mouse monoclonal anti-neuronal specific nuclear protein (NeuN, neuronal marker,

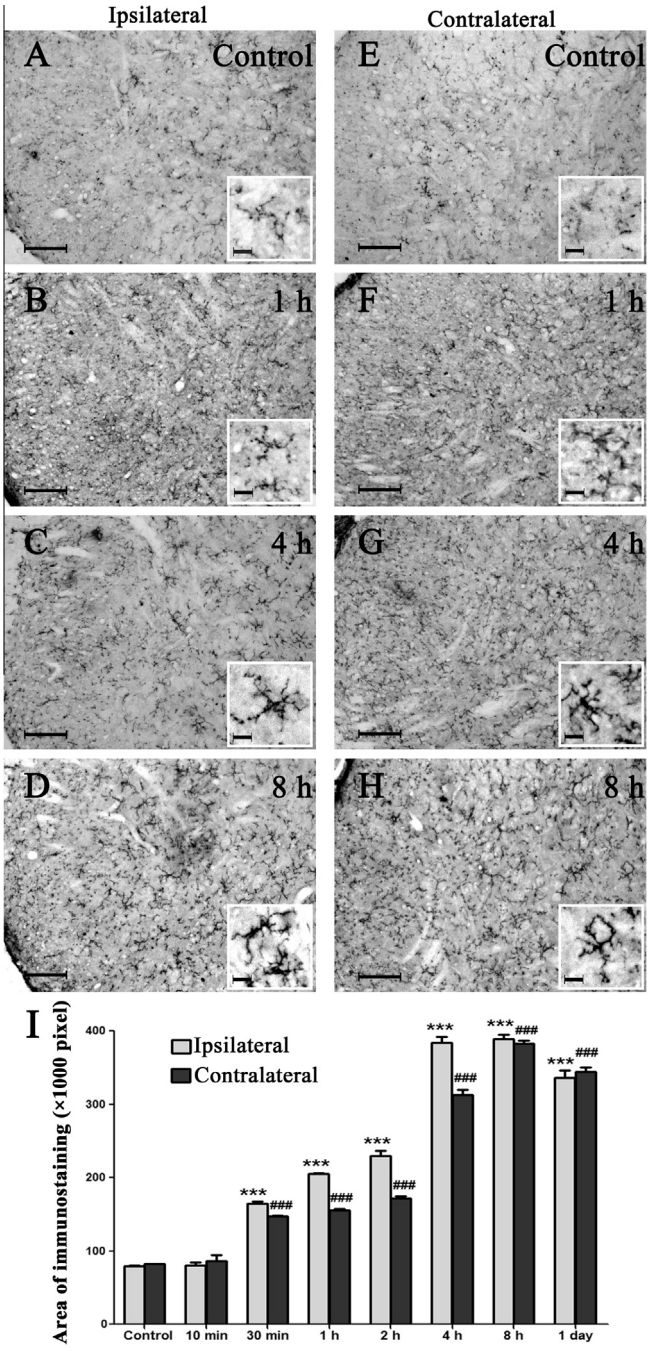

Fig. 1. Microglial activation in the ipsilateral (A–D) and the contralateral (E–H) spinal cord after i.pl. BmK I injection. A & E showed microglial morphology in saline-treated animals. Other pictures showed OX-42 immunostaining at each time point after BmK I injection. (I) Statistical histogram of OX-42 immune-reactivity in L4–L5 spinal cord. $^{***}p < 0.001$, ipsilateral side compared with control group; $^{###}p < 0.001$, contralateral side compared with control group. Scale bar: 200 μm. Scale bar for insets: 50 μm.

1:500; Millipore Bioscience Research Reagents), goat biotinylated anti-rabbit or goat biotinylated anti-mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for ABC method immunohistochemistry, and goat anti-rabbit fluorescein indocarbocyanine-conjugated or goat anti-mouse isothiocyanate-conjugated secondary antibody (1:200; Jackson Immuno Research Inc., West Grove, PA).

2.5. Behavioral tests

In the study, behavioral tests were used to evaluate the suppressive effect of SB203580 on BmK I-induced pain responses. The measurement of paw withdrawal mechanical threshold (PWMT) and paw withdrawal thermal latency (PWTL) followed the methods described by Bai et al. [23].

In brief, the rats were placed on a mesh floor. The mechanosensitivity was evaluated by a series of von Frey fibers with forces range of 0.6–26 g (Stoelting Co., IL). Fibers were applied to rat hind paws for the same duration of 2–3 s with an interval of 10 s. The positive response is indicated by brisk withdrawal or flinching of the tested hand paw. The rat PWMT was defined as the lowest force that caused at least five withdrawals out of the ten consecutive applications For the measurement of PWTL, rats were placed on the surface of a transparent glass plate. Steady heat stimuli were projected with a radiant heat stimulator targeting the palm of hind paw. The cutoff time was set as 20 s to prevent tissue injury. For each rat, five stimuli were applied with an interval of 10 min. The PWTL value was determined by averaging the three middle values.

2.6. Statistical analysis

All results were expressed as mean ± S.E.M. (standard error of the mean). Data of immunohistochemical staining were compared by One-way ANOVA followed by a Bonferroni's post hoc test between each groups. Two-way ANOVA followed by a Bonferroni's post hoc test was used to analyze the suppression of SB203580 on pain behaviors.

3. Results

3.1. I pl. injection of BmK I induces proliferation and activation of glial cells in the spinal cord

In our previous study, we evaluated the changes of sensitivity to mechanical and heat stimuli in bilateral rat hind paw. It was found that the rat bilateral PWMT value decreased dose-dependently from 2 h to 7 days after BmK I injection. The mechanosensitivity reached the peak around 2 h to 1 day. Similarly, the rat PWTL value in the injected hand paw also decreased from 4 h to 72 h, and reach the valley from 4 h to 8 h [23]. Referring to the behavioral time course, immune staining of OX-42 and GFAP was performed to assess the spinal activation of microglia cells and astrocytes in the present study. OX-42 immunoreactivity in the L4–L5 spinal cord at various time points following the delivery of BmK I, by administration of i.pl. injection, was captured, as shown in Fig. 1. In comparing the naive (Fig. 1 A and E) to the 10 min-exposed group groups, microglia showed small soma with thin branched processes in both. However, the i.pl. intensity of OX-42 increased gradually from 30 min to 24 h post-BmK I administration (Fig. 1 B–D). Activated microglia exhibited marked morphological changes including increased cell size and thickened branches. Similar changes were observed in the contralateral side (Fig. 1 F–H). Statistical analysis reveals that OX-42 immunoreactivity in the bilateral spinal cord began to increase at 30 min ($p < 0.001$, compared with control group), with a peak at 8 h ($p < 0.001$, compared with control group) and gradually recover 1 day after BmK I application (Fig. 1I).

No significant difference of bilateral GFAP immunoreactivity was found in the L4–L5 spinal cord dorsal horn at different time points (Fig. S2 A–G).

3.2. Activation of p38 in the spinal cord of BmK I-inflamed rats

To determine the relationship between i.pl. injection of BmK I and p38 MAPK activation, immunohistochemistry was employed

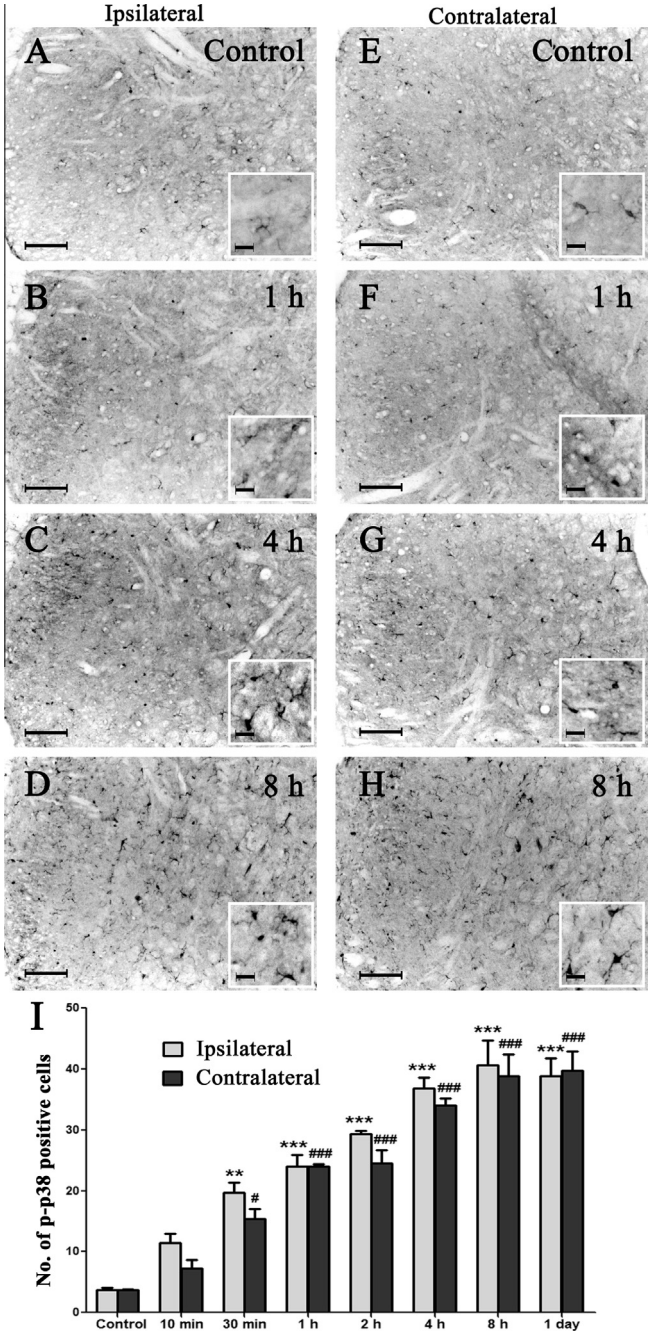

Fig. 2. The time course of p38 activation in the ipsilateral (A–D) and the contralateral(E–H) spinal cord after i.pl. BmK I injection. A and E showed p-p38 immune-reactivity in saline-treated animals. Other micrographs showed p-p38 immunostaining at each time point after BmK I injection. (I) Statistical histogram of p-p38 immune-reactivity in L4–L5 spinal cord dorsal horn. $^{**}p < 0.01$, $^{***}p < 0.001$, ipsilateral side compared with control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, contralateral side compared with control group. Scale bar: 200 μm. Scale bar for insets: 50 μm.

to examine the phosphorylation level of p38 in L4–L5 spinal cord at different time points (Fig. 2). A low level of p-p38 was observed in naive or saline injected groups (Fig. 2 A and E). The number of p-p38 immune-reactive (I.R.) cells began to increase 30 min post-BmK I administration (ipsilateral, $p < 0.01$, Fig 2B–D; contralateral, $p < 0.05$, Fig 2F–H), and peaked at 8 h on both sides of the spinal cord ($p < 0.001$, Fig. 2I).

3.3. Cellular localization of phosphorylated p38 post i.pl. BmK I administration

Double-labeled immunofluorescence staining was performed to identify the cell types that expressed p-p38 in the spinal cord after BmK I administration. p38 activity was shown to be minimal in both astrocytes (Fig. 3A–C) and in spinal neurons (Fig. 3G–I) during the examined time interval, whereas the majority of p-p38 was well localized in OX-42-positive microglia (Fig. 3D–F).

3.4. Suppression of SB203580 on microglial activation

As microglial distribution of p-p38 is specific, we wondered whether the activation of microglia is a p38-dependent process. SB203580 or saline was i.t. delivered before BmK I injection. The OX-42 immunoreactivity in L4–L5 spinal cord at different time points was captured and quantified (Fig. 4A–F). Compared to the control group, a sharp reduction of microglial activation in both sides of the spinal cord was observed in SB203580-treated group.

3.5. Suppression of SB203580 on pain-related behaviors

Bilateral mechanical hypersensitivity induced by BmK I was inhibited significantly following pretreatment with SB203580 at 4 and 8 h time points. Low (2 μg) and moderate (10 μg) doses of SB203580 exerted partial suppression on BmK I-induced bilateral mechanical hypersensitivity. A high dose (50 μg) of SB203580 markedly attenuated the development of mirror-image mechanical hypersensitivity (Fig. 4 G and H). Ipsilateral PWMTs were increased by intrathecal 2, 10 and 50 μg SB203580 from 2.0 ± 0 g to 5.0 ± 0.93 g ($p < 0.01$), 5.0 ± 0.38 g ($p < 0.01$), 7.75 ± 0.59 g ($p < 0.001$) at 4 h, and PWMT was increased by intrathecal 50 μg SB203580 from 5.25 ± 0.37 g to 11.875 ± 0.91 g ($p < 0.001$) at 8 h. Contralateral PWMT were increased by intrathecal 50 μg SB203580 from 13.75 ± 0.82 g to 23.25 ± 1.80 g ($p < 0.01$) at 4 h, and PWMTs were increased by intrathecal 10 and 50 μg SB203580 from 15.125 ± 1.75 g to 23.25 ± 1.80 g ($p < 0.05$) and 26 ± 0 g ($p < 0.01$) at 8 h.

Similarly, BmK I-induced ipsilateral thermal hypersensitivity was inhibited significantly by pretreatment with SB203580 at 4 and 8 h timepoints. Neither SB203580, nor 25% DMSO treatment alone, could alter the contralateral paw thermal withdrawal latency (Fig. 4J). All the three doses of SB203580 (2, 10, or 50 μg) attenuated the development of the ipsilateral thermal hypersensitivity (Fig. 4I). Ipsilateral PWTLs were increased by intrathecal 2, 10, and 50 μg of SB203580 from 7.4 ± 0.55 s to 10.4 ± 0.63 s ($P < 0.05$), 10.6 ± 0.54 s ($p < 0.05$), and 12.6 ± 0.49 s ($p < 0.001$) at 4 h, and PWTL was increased by intrathcal 50 μg of SB203580 from 9.8 ± 0.42 s to 13.5 ± 0.47 s ($p < 0.01$) at 8 h.

No significant differences were observed between 25% DMSO- and saline-treated groups in all of the above tests (data not shown).

4. Discussions

Activation of spinal microglia has been found in many animal pain models including formalin test, spinal nerve ligation, and sciatic nerve inflammation [24–26]. In this study, we found that both sides of the spinal microglia were activated by peripheral application of BmK I, with the contralateral activation gradually achieving an equivalent level with the ipsilateral side. As we all known, activated microglia produced numerous inflammatory factors under pain state [5]. Moreover, according to the blood transmission theory, blood can be a medium to convey inflammatory factors to the uninjured side, thereby inducing a mirror-image of pain symptoms

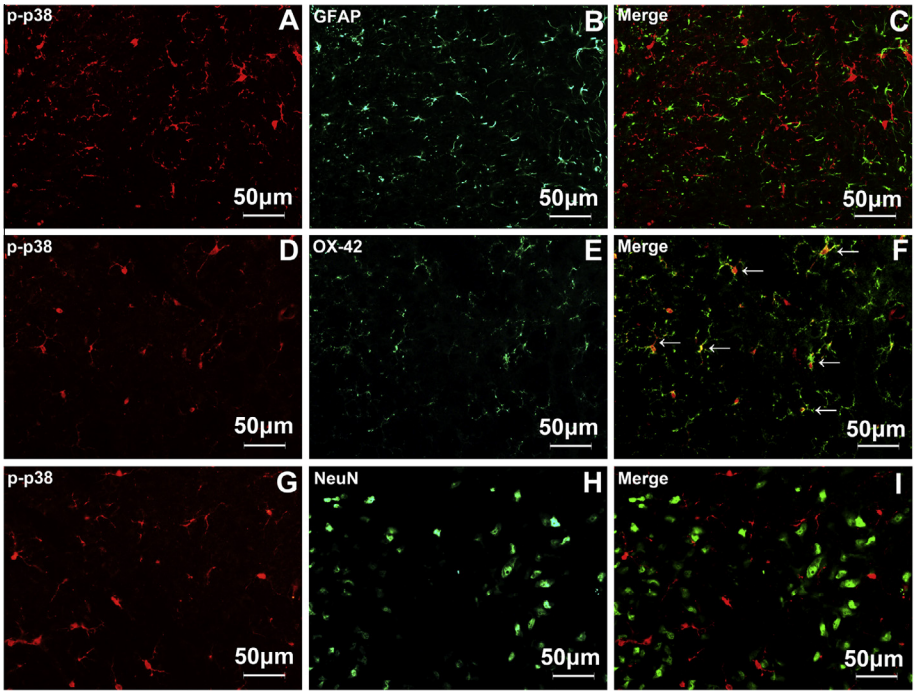

Fig. 3. Representative photomicrographs of double-labeling histochemistry 8 h after i.pl. BmK I injection. Images illustrate co-localization of p-p38 in spinal neuron, microglia and astrocyte. The immunolabel is shown in the pictures. Almost all positive signal was co-localized with OX-42 labeled microglial cells (D–F).

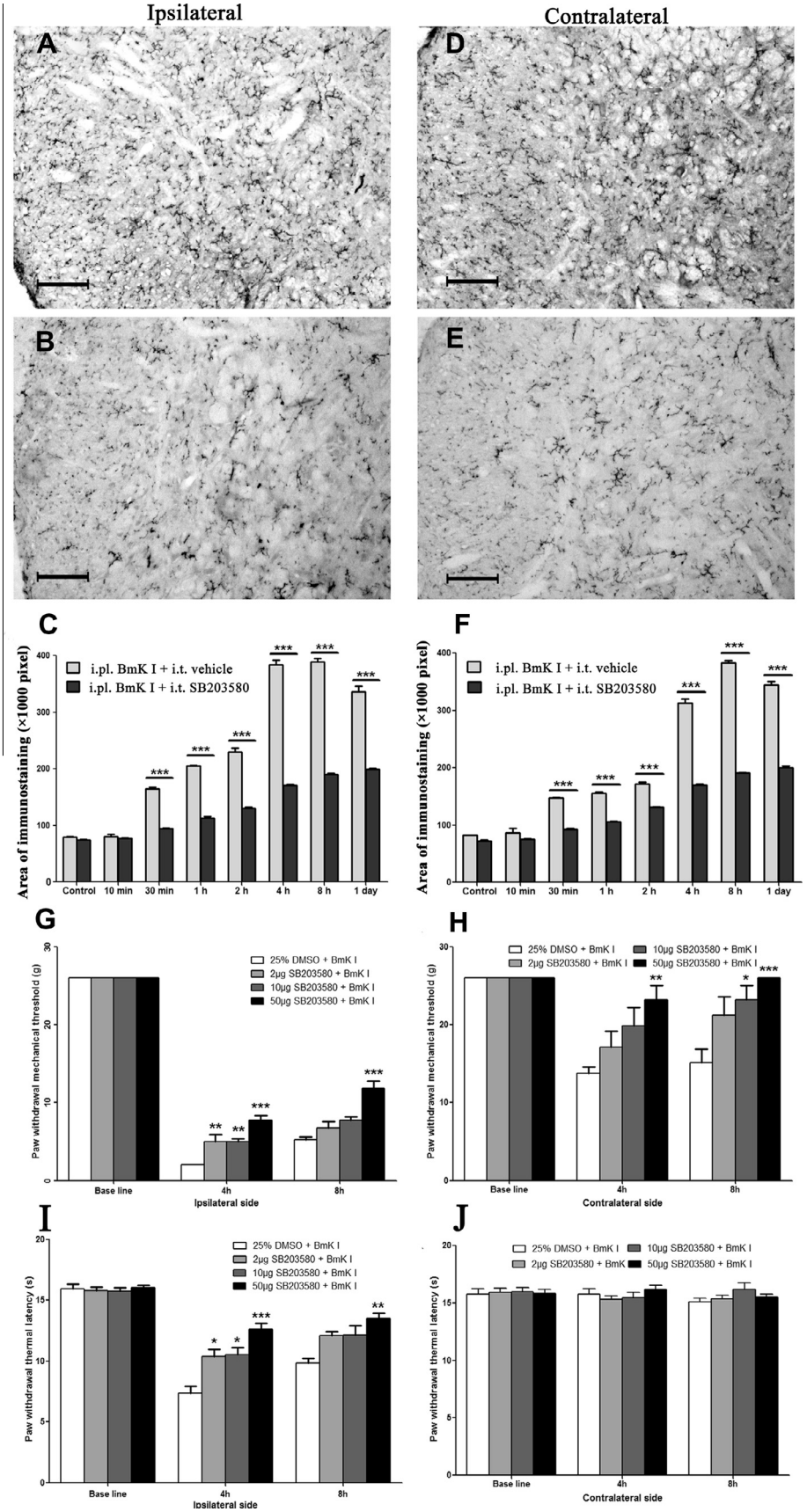

Fig. 4. Systemic administration of SB203580 suppressed BmK I-induced microglial activation and reduced BmK I-induced pain-related behaviors. (A and D) shows the morphology of activated microglia in vehicle-treated animals, while (B and E) shows that the activation of microglia was significantly suppressed by SB203580 in both sides of spinal cord dorsal horn. Scale bar: 200 μm. Statistical histogram of OX-42 immune-reactivity in ipsilateral (C) and contralateral (F) L4–L5 spinal cord after i.t. SB203580 injection. Bilateral mechanical hypersensitivity (G and H) and ipsilateral thermal hypersensitivity (I) were suppressed. All behavioral data was presented as mean ± S.E.M., $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with vehicle-treated group.

[27]. However, in this study, OX-42 positive cells were also observed near the myelocoele. This anatomical localization provided another possibility that the pain signals were directly delivered to the contralateral side by the activated microglia and subsequently induced the mirror-image mechanical hypersensitivity.

The activation of astrocytes following peripheral nerve or tissue injury was thought to contribute to the maintenance of pain in a number of chronic pain models [28]. Previously, it was found that astrocytes in the bilateral spinal cord could be activated at day 3, with a peak at day 7, following i.pl. injection of BmK venom [29]. The present study captured the OX-42 and GFAP positive cells from 10 min to 24 h post-BmK I administration, which is thought to be the key period of development and maintenance of BmK I-induced hyperalgesia. To date no significant evidence suggests astrocytes are activated in this time scale (Fig. S2). Thus, the role of astrocyte in BmK I-induced hyperalgesia requires further study.

Increasingly, evidence suggests that the activation of p38 in the spinal cord dorsal horn and dorsal root ganglion contributed to the development and maintenance of inflammatory [30] and neuropathic pain [9]. The synthesis of numerous inflammatory factors could be regulated by activated p38 pathway in both transcriptional and posttranslational level [31]. The present study showed that the inhibition of p38 suppressed hyperalgesia reflex induced by BmK I, demonstrating that phosphorylation of p38 contribute to scorpion envenomation pain.

Furthermore, the activated p38 was only detected in spinal microglia, but not noted to display activity in the neurons nor among astrocytes. There was a parallel activation time course between p38 and microglia. Interestingly, SB203580 (the specific inhibitor of p-p38) significantly suppressed the activation of spinal microglia as indicated at 30 min post-i.pl. injection of BmK I. This suggests that there is a definitive relationship of p38 signaling pathway in nociception-related spinal microglial activation.

The peripheral and central nervous system mechanisms underlying BmK I-induced hyperalgesia have been investigated in previous studies [23,32]. However, the role of spinal microglia in BmK I-induced central sensitization remains unclear, as the mediators released from the microglia can create a feedback loop to itself [33] for sustained activation once initiated. In other words, in response to the stimuli, microglia can continuously sensitize the central nerve system. The i.pl. injection of BmK I elicited a violent peripheral and central sensitization response which was reflected as rat pain-related behaviors. Behavioral data in this work suggests that the p38 signal pathway may be positioned in a critical location to facilitate BmK I-induced nociceptive responses. In summary, the present study demonstrates that p38-dependent spinal microglia activation is responsible for the maintenance of scorpion envenomation pain responses. Understanding p38 signaling in microglia will greatly improve our knowledge to the regulation of pain resulting from scorpion envenomation. As evidence of the importance of p38 in pain regulation, several p38 inhibitors are currently under clinical trials to determine their effectiveness in the relief of pain and inflammatory diseases [34].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.071.

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