Inhibition of Mitogen-Activated Protein Kinases Phosphorylation Plays an Important Role in the Anti-nociceptive Effect of Pregabalin in Zymosan-Induced Inflammatory Pain Model

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Although pregabalin has been shown to have preclinical and clinical efficacy in neuropathic pain, the mechanism of its antinociceptive action is still unknown in other pain states. This study aimed to evaluate the antinociceptive effect of pregabalin and its underlying spinal mechanisms related to mitogen activated protein kinases (MAPKs) in neuron and microglia following intraplantar injection of zymosan model. Zymosan evoked thermal hyperalgesia, mechanical hyperalgesia, and mechanical allodynia starting from 1 h and persistent until 5 h post-injection, which were dose-dependently reversed by oral pretreatment of pregabalin (3, 10, and 30 mg/kg). Pregabalin dramatically inhibited zymosan-induced Fos expression (a marker for neuronal activation) and microglia activation (using markers CD11b and ED1) in the spinal dorsal horn. Moreover, zymosan significantly increased phosphorylation of extracellular signal-regulated protein kinase (ERK) 1/2 (double labeling with neuron), ERK5 (double labelling with neuron and microglia) and p38 MAPK (double labeling with microglia) in the spinal dorsal horn, which overall elevations were reversed by pregabalin. These findings suggest that blockage of MAPKs activation in neuron and microglia might be closely related to the antinociceptive effect of pregabalin on zymosan-induced peripheral inflammatory pain.

Key words pregabalin; pain; mitogen activated protein kinase (MAPK); zymosan

Pregabalin as a new-generation anti-epileptic drug with fewer side effects and less tolerance has been shown to have preclinical and clinical efficacy in neuropathic pain. Accumulative clinical studies in different types of neuropathic pain (i.e., peripheral diabetic neuropathy, fibromyalgia, postherpetic neuralgia, cancer chemotherapy-induced neuropathic pain) have demonstrated its effectiveness in treating the neurological symptoms including allodynia and hyperalgesia.1) In several type of neuropathic pain models, it has been further demonstrated that pregabalin has an influence on various neuronal targets and the signaling systems including calcium channel-mediated neurotransmitter release and activation of excitatory amino acid pathway in the spinal level.2) Despite widespread clinical use and characterization of antinociceptive activity of pregabalin in neuropathic pain, potential antinociceptive effect and its underlying molecular mechanisms in other pain state remain poorly understood. Thus, present study was focus on the potential antinociceptive effect of pregabalin in the peripheral inflammatory pain state.

In recent years, microglia has been increasingly implicated in the mechanisms underlying abnormal pain states. Spinal microglia activation (using markers CD11b and EDI) has been observed in the early phase of neuropathic pain,2) which is closely correlated with induction and maintenance of allodynia and hyperalgesia.3) In line with this view, microglia inhibitors successfully alleviate neuropathic pain in experimental models.3,4) Similar with neuropathic condition, peripheral inflammation by intraplantar zymosan injection as an experimental rheumatoid arthritis model also results in the development of hyperalgesia and allodynia accompany with spinal microglia activation and cytokine expression.5,6) Accumulative data have been revealed that activation of mitogen activated protein kinases (MAPKs, i.e., extracellular signal-regulated protein kinase (ERK) 1/2, p38 MAPK, and ERK5) in both spinal neurons and microglia may have a pivotal role in development of pain symptom following nerve injury and peripheral inflammation.7–9) In this context, we aimed to evaluate whether the spinal activation of MAPKs signaling pathway in both neuron and microglia is involved in the antinociceptive effect of pregabalin in zymosan induced peripheral inflammatory pain model.

METHODS

Animals Male Sprague-Dawley rats (7 weeks, 200–220 g, Dae Han Biolink Co., Eumsung, South Korea) were housed in colony cages with free access to food and water and maintained in temperature and light controlled rooms (23±2°C, 12/12 h light/dark cycle with lights on at 08:00) at least 1 week before experiment. All of the methods used in the present study were approved by the Institute of Animal Care and Use Committee at Chonbuk National University and conform to NIH guidelines (NIH Publication No. 86-23, revised in 1985).

Inflammation and Drug Treatment Inflammation was induced by a single subcutaneous injection (6 mg/200 µL in saline) into the plantar surface of the right hind paw of zymosan (Sigma, MO, U.S.A.) under anesthetized with 3% isoflurane in a mixed N2O/O2 gas as previously described.6) Animals were assigned 6 animals for each experimental group (0, 3, 10, and 30 mg/kg of pregabalin). Pregabalin (Tocris, Bristol, U.K.) was dissolved in saline and orally administrated at 1 h before zymosan injection. We selected the highest dose of pregabalin (30 mg/kg), which dose began obvious increase of sleepy behavior in most animals as a predictable side effect.

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Pain Behavioral Assay Animals were acclimatized to the testing chambers for 1 h at 1 d before and the day of inflammation induction. Measurement of each pain behavioral test was blindly and performed at before (at 0 h as basal level) and 1, 3, and 5 h after zymosan injection. Thermal hyperalgesia (Thermal plantar tester, Ugo Basile, Varese, Italy), mechanical hyperalgesia (Analgesy-meter, Ugo Basile) and mechanical allodynia were evaluated as previously described.10) To assess mechanical allodynia, a series of eight von Frey hair monofilaments (0.4, 0.6, 1, 2, 4, 8, 15, and 20 g, Stöelting, IL, U.S.A.) were applied to the middle of the middle and fourth toe of the hind paw for a maximum of 3 s, or until the animal displayed a nociceptive response comprised of paw lifting and/or shaking. Testing was initiated with the 2 g filament.

Immunohistochemistry Finishing behavioral observation at 5 h after zymosan injection, three rats of each group showing average value in each pain test was selected and deeply anesthetized with 5% isoflurane and perfused transcardially with calcium-free Tyrode’s solution followed by fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffered saline (PBS, pH 6.9). L4–5 spinal cord was removed immediately after perfusion, post-fixed in the same fixative for 4 h and then cryoprotected in PBS containing 30% sucrose (pH 7.4). A series of frozen sections (40 µm thickness in spinal cord) was cut using a cryostat. Preblocking with 5% appropriate normal serum and 0.3% triton X-100 in PBS, the sections were incubated in rabbit anti-Fos (Merck, Darmstadt, Germany, 1:10000), mouse anti-CD11b (Serotec, Oxford, U.K., 1:10000), mouse anti-NeuN (Millipore, 1:10000), rabbit anti-ED1 (Cell Signaling, MA, U.S.A., 1:1000), rabbit anti-phosphorylated-ERK1/2 (p-ERK1/2, Cell Signaling, 1:1000), rabbit anti-phosphorylated-ERK5 (p-ERK5, Cell Signaling, 1:1000), or rabbit anti-phospho-p38 mitogen-activated protein kinase (p-p38, Cell Signaling, 1:500) at 4°C for 2 d. The sections were subsequently incubated in Alexa fluor568-conjugated host specific secondary antibody (Invitrogen, CA, U.S.A., 1:200) for 2 h at room temperature. For double immunofluorescent staining, sections were firstly incubated with primary antibody and visualized with Alexa Fluor488 conjugated secondary antibody (1:200, Invitrogen, CA, U.S.A.) and the other
primary antibody was visualized with Alexa fluor568. After coverslipping, spinal tissues were scanned with ECLIPSE 80i (Nikon, Japan) fluorescent microscope, and five sections were selected from each animal. Images of individual sections were digitized with 4096 grayscale levels using a cooled CCD camera (CoolSnap ES, Roper, Japan) connected to a computer-assisted image analysis system (Metamorph, Universal Imaging Co., West Chester, PA, U.S.A.). In order to maintain a constant threshold for each image and to compensate for subtle variability in the immunostaining, we only calculated positive immunoreactivity as threshold area that are at least 200% brighter than the average grayscale level of each image.

Statistical Analysis Data values were expressed as the mean±S.E.M. Behavioral data were analyzed using the commercially available software GraphPad Prism 6.0 (Graphpad Software, CA, U.S.A.). Statistical analysis was carried out using two-way ANOVA for repeated measures followed by Dunnett’s Multiple Comparison test.

RESULTS

Rat that received intraplantar zymosan injection exhibited typical pain behaviors including thermal hyperalgesia (Fig. 1A), mechanical hyperalgesia (Fig. 1B), and mechanical allodynia (Fig. 1C) during 5h observation period. Time course nociceptive responses in pregabalin treated groups (10 or 30mg/kg) were significantly decreased zymosan induced thermal hyperalgesia (Fig. 1A, *p<0.05, **p<0.01 as compared to those in saline-treated group; group factor: F(3, 15)=11.21, p=0.0004; time factor: F(3,15)=210.2, p<0.0001). Zymosan induced mechanical hyperalgesia was dose-dependently inhibited by pregabalin (10 or 30mg/kg) pretreatment (Fig. 1B, *p<0.05, **p<0.01 as compared to those in saline-treated group; group factor: F(3, 15)=38.85, p<0.0001; time factor: F(3,15)=430.0, p<0.0001). Dose-dependent anti-nociception of pregabalin was also observed in zymosan induced mechanical allodynia (Fig. 1C, *p<0.05, **p<0.01 as compared to those in saline-treated group; group factor: F(3, 15)=38.49, p<0.0001; time factor: F(3,15)=217.3, p<0.0001).

Zymosan significantly increased Fos expression as neuronal pain marker as compared with normal animals in the ipsilateral spinal lamina I–II, which was completely normalized by the pretreatment of pregabalin (30mg/kg, Figs. 1E–G, image analysis data: Fig. 1D). On the other hand, zymosan also produced the activation of microglia confirmed by CD11b (Figs. 2A–C) and ED1 (Figs. 2D, F) immunoreactivity in the ipsilateral spinal lamina I–IV, which was revered by pregabalin (30mg/kg). Zymosan induced inflammation significantly increased the expression of pERK1/2 (Figs. 3A, B) and pERK5 (Figs. 3D, E) in the spinal lamina I–II. Although pERK1/2 was distinctly merged with spinal neuron (using NeuN) rather than microglia (Figs. 3J, K), pERK5 was merged with neuron as well as microglia (Figs. 3L, M). On the other hand, the expression of p-p38 was increased after zymosan injection in the spinal lamina I–IV (Figs. 3G, H), which was distinctly co-localized with microglia (Figs. 3N, O). Finally, we observed that pregabalin treatment dramatically reduced zymosan induced spinal MAPKs activation including pERK1/2 (Fig. 3C), pERK5 (Fig. 3F) and p-p35 (Fig. 3I).

DISCUSSION

In current behavioral studies using intraplantar zymosan injection, hyperalgesia and allodynia develop to mechanical or thermal stimuli, which was dose-dependently attenuates pregabalin pretreatment. Accompanying with behavioral test, zymosan induced Fos elevation as a marker for neuronal activation in spinal lamina I–II was also reduced by pregabalin. There is accumulative evidence indicating that activation of MAPK in both primary afferents and the spinal cord may participate in the establishment and maintenance of nociceptive-induced plasticity.7–9 Present study show that zymosan induced inflammation significantly increased phosphorylation of MAPKs including ERK1/2 and ERK5 in the spinal dorsal
The ERK1/2 is activated by membrane depolarization and calcium influx, and activated by an upstream kinase, MAPK/ERK kinase (MEK). Although a MEK inhibitor U0126 produces similar antinociceptive effect in both neuropathic pain and inflammatory pain, the distinct activation of ERK in the primary afferents occurs in different populations of dorsal root ganglion (DRG) neurons after peripheral inflammation (small to medium diameter DRG) and nerve injury (medium to large diameter DRG), respectively. In addition, complete Freund’s adjuvant (CFA) induced inflammation evokes ERK5 expression in both DRG and spinal cord, and ERK5 knockdown significantly reduces pain and spinal Fos elevation. Support to these findings, we observed that the elevated phosphorylation of ERK1/2 and ERK5 by zymosan detected in spinal lamina I–II, which was similar location of zymosan induced Fos expression. Subsequent double labeling study further confirmed zymosan induced ERK1/2 and ERK5 phosphorylation occurred in spinal neurons. Moreover, present study demonstrated that pretreatment of pregabalin dramatically reversed zymosan induced spinal activation of MAPKs including ERK1/2 and ERK5, which may be potential targets for pharmacological intervention of pregabalin. As a major pharmacological mechanism, it is well known that pregabalin specifically binds to alpha-2-delta subunit in primary afferent fiber and reduces glutamate release into spinal cord. Moreover, accumulative data has been fully addressed that activation of ionotropic and metabotropic glutamate receptors is closely linked with the MAPK activation. Although we did not perform the additional intrathecal study of pregabalin, it is reasonable to assume that oral treatment of pregabalin as a major clinical treatment route may produce antinociceptive effect through inhibition of MAPK in the spinal level.

Spinal microglial activation might be just the first step in a cascade of central nervous system immune responses following injury and inflammation. In neuropathic pain model, it is well documented that microglial activation (using markers CD11b and ED1) accompanying p38 phosphorylation in the spinal cord plays an important role in the development of pain. Current study also revealed that zymosan dramatically increased spinal p38 phosphorylation as well as microglia marker (CD11b).
lia proliferation (CD11b) and phagocytic marker (ED1) elevation in the spinal dorsal horn. It was notable that zymosan induced phosphorylation p38 exclusively observed in microglia but not in spinal neurons in present double labelling study. Moreover, we found that pregabalin significantly reduced zymosan induced spinal p-38 activation and microglia proliferation. Both peripheral inflammation and nerve injury induces p38 activation in spinal microglia, and p38 inhibitor SB203580 reduces inflammation-induced thermal hyperalgesia and spinal nerve ligation-induced mechanical allodynia. Moreover, intrathecal injection of minocycline that inhibits spinal microglia activation by blockade of p38 produces antinociceptive effect in ether inflammation or neuropathic pain model. On the other hand, we found that zymosan induced elevation of ERK5 phosphorylation also observed in spinal microglia which was reversed by pregabalin. It is notable that antisense knockdown of ERK5 suppressed nerve injury-induced neuropathic pain and decreased microglial activation. Therefore, present data suggested that the inhibitory effect on microglial p-38 and ERK5 activation may be responsible for the antinociceptive effect of pregabalin under inflammatory conditions.

In conclusion, present study demonstrated that pregabalin produced the antinociceptive effect on zymosan induced inflammatory hyperalgesia and allodynia. Moreover, the antinociceptive mechanisms of pregabalin are closely associated with the inhibitory role of MAPK activation in both spinal neuron (p-ERK1/2 and p-ERK5) and microglia (p-ERK5 and p-p38) under inflammatory condition.

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REFERENCES

1) Verma V, Singh N, Jaggi A. Pregabalin in neuropathic pain: evidences and possible mechanisms. Curr. Neuropharmacol., 12, 44–56 (2014).
2) Hu P, Bembrick AL, Keay KA, McLachlan EM. Immune cell involvement in dorsal root ganglia and spinal cord after chronic constriction or transection of the rat sciatic nerve. Brain Behav. Immun., 21, 599–616 (2007).
3) Graeber MB, Christie MJ. Multiple mechanisms of microglia: A gatekeeper’s contribution to pain states. Exp. Neurol., 234, 255–261 (2012).
4) Scholz J, Abele A, Marian C, Haussler A, Herbert TA, Woolf CJ, Tegeder I. Low-dose methotrexate reduces peripheral nerve injury-evoked spinal microglial activation and neuropathic pain behavior in rats. Pain, 138, 130–142 (2008).
5) Sweitzer SM, Colburn RW, Rutkowski M, DeLeo JA. Acute peripheral inflammation induces moderate glial activation and spinal IL-1beta expression that correlates with pain behavior in the rat. Brain Res., 829, 209–221 (1999).
6) Meller ST, Gebhart GF. Intraplantar zymosan as a reliable, quantifiable model of thermal and mechanical hyperalgesia in the rat. Eur. J. Pain, 1, 43–52 (1997).
7) Obata K, Noguchi K. MAPK activation in nociceptive neurons and pain hypersensitivity. Life Sci., 74, 2643–2653 (2004).
8) Xiao C, Zhang L, Cheng QP, Zhang LC. The activation of extracellular signal-regulated protein kinase 5 in spinal cord and dorsal root ganglia contributes to inflammatory pain. Brain Res., 1215, 76–86 (2008).
9) Katsura H, Obata K, Mizushima T, Sakurai J, Kobayashi K, Yamamura H, Dai Y, Fukuoka T, Sakagami M, Noguchi K. Activation of extracellular signal-regulated protein kinases 5 in primary afferent neurons contributes to heat and cold hyperalgesia after inflammation. J. Neurochem., 102, 1614–1624 (2007).
10) Kim SH, Kwon JK, Kwon YB. Pain modality and spinal glia expression by streptozotocin induced diabetic peripheral neuropathy in rats. Lab. Anim. Res., 28, 131–136 (2012).
11) Matsuzawa R, Fujiwara T, Nemoto K, Fukushima T, Yamaguchi S, Akagawa K, Hori Y. Presynaptic inhibitory actions of pregabalin on excitatory transmission in superficial dorsal horn of mouse spinal cord: further characterization of presynaptic mechanisms. Neurosci. Lett., 558, 186–191 (2014).
12) Wang JQ, Fibuch EE, Mao L. Regulation of mitogen-activated protein kinases by glutamate receptors. J. Neurochem., 100, 1–11 (2007).
13) Shi XQ, Zekki H, Zhang J. The role of TLR2 in nerve injury-induced neuropathic pain is essentially mediated through macrophages in peripheral inflammatory response. Glia, 59, 231–241 (2011).
14) Cao H, Zhang YQ. Spinal glial activation contributes to pathological pain states. Neurosci. Biobehav. Rev., 32, 972–983 (2008).
15) Obata K, Katsura H, Mizushima T, Sakurai J, Kobayashi K, Yamamura H, Dai Y, Fukuoka T, Noguchi K. Roles of extracellular signal-regulated protein kinases 5 in spinal microglia and primary sensory neurons for neuropathic pain. J. Neurochem., 102, 1569–1584 (2007).