Sigma-1 Receptor Antagonist, BD1047 Reduces Nociceptive Responses and Phosphorylation of p38 MAPK in Mice Orofacial Formalin Model

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Sigma-1 receptors (Sig-1Rs) play a role in different types of pain and in central sensitization mechanisms in spinal cord. However, it is currently unexplored whether Sig-1Rs are involved in orofacial pain processing. Here we show whether a selective Sig-1R antagonist, BD1047 reduces nociceptive responses in the mouse orofacial formalin model and the number of Fos-immunoreactive (ir) cells in the trigeminal nucleus caudalis (TNC). In addition, the phosphorylation of extracellular signal-regulated kinase (pERK) or p38 (pp38) mitogen-activated protein kinases (MAPK), which are closely linked to pain signaling and sensitization, in TNC was modified by BD1047.

The orofacial region is one of the most densely innervated, by the trigeminal nerve, areas of the body, which focuses some of the most common acute pains, i.e., those accompanying the pathological states of the teeth and the related structures. It is also the site of frequent chronic post-herpetic neuralgia, migraine, and referred pains. However, the neurophysiological mechanisms underlying these orofacial pains are still poorly understood. In addition, only few analgesic trials have been undergone in trigeminal region, and a lot of difficulties in the management of acute and chronic orofacial pain conditions stem from a lack of recognition and understanding of pain mechanisms. Thus, the management of orofacial disorders is one of the most challenging in the pharmacology field in relation to analgesics.

It is well recognized that sigma-1 receptors (Sig-1Rs) are widely distributed in mammalian central nervous system (CNS) including certain cortical areas, the hypothalamus and the dorsal horn of the spinal cord. In addition, Sig-1Rs play an important role in a variety of cellular functions via modulation of intracellular Ca2+ concentration. Cumulative evidences from several researchers including our laboratories have shown that Sig-1Rs have a pronociceptive effect in acute and chronic pain condition using the formalin-induced pain test and sciatic nerve injury-induced pain animal models. In addition, we have examined that the direct activation of Sig-1Rs in spinal cord enhances the response to peripheral mechanical and thermal stimuli via Ca2+-dependent second messenger cascades. However, it is unclear whether the activation of Sig-1Rs is involved in orofacial pain processing.

Therefore, the present study was designed to verify whether a selective Sig-1R antagonist, BD1047 could reduce nociceptive responses in the mouse orofacial formalin model and the number of Fos-immunoreactive (ir) cells in the trigeminal nucleus caudalis (TNC). In addition, we examined whether the phosphorylation of extracellular signal-regulated kinase (pERK) and p38 (pp38) mitogen-activated protein kinases (MAPK), which are closely linked to pain signaling and sensitization, in TNC were modified by BD1047 treatment.

MATERIALS AND METHODS

Animals The experiments were performed using male C57BL/6 mice (25–30 g; Central Lab. Animal Inc., Seoul, Korea) 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 07:00) for at least 1 week prior to the experiment. The experimental protocols for animal usage were reviewed and approved by the Kyung Hee University Institutional Animal Care and Use Committee and conformed to National Institutes of Health guidelines (NIH publication No. 86-23, revised 1985).

Formalin-Induced Orofacial Pain Test Formalin was prepared from commercially available stock formalin (aqueous solution of 37% formaldehyde, Sigma, St. Louis, MO, U.S.A.) and further diluted in physiological saline to 5%. Behavioral experiments were conducted in a quiet behavioral testing room. Mice were first acclimatized for 30 min in an acrylic observation chamber (15×15×15 cm). Following the acclimatization, mice were received a 10 µL subcutaneous injection of 5% formalin through a 30-gauge needle attached.
to Hamilton syringe into the right upper lip, just lateral to the nose. Following injection, the animals were immediately placed back in the test chamber and nociceptive responses in each animal were recorded using a video camera for a 45 min observation period. The recording time was divided into 15 blocks of 3 min, and a nociceptive score was determined for each block by measuring the number of seconds that the animals spent grooming the injected area with the ipsilateral fore- or hindpaw. Movements of the ipsilateral forepaw were accompanied by movements of the contralateral forepaw. The duration of the responses during the first 2 blocks represented the first phase (0–6 min post-injection), while the duration of responses during subsequent 13 blocks represented the second phase (6–45 min post-injection) in the formalin-induced orofacial pain test. Analysis of the behavior was made by an investigator who was blinded to the animal's group assignment. Sig-1R receptor antagonist, BD1047 (N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine dihydrobromide, Tocris, Bristol, U.K.) was diluted in physiological saline. For assessment of its effect on formalin-induced orofacial pain, BD1047 at a dose of 1, 3, or 10 mg/kg was intraperitoneally (i.p.) injected 30 min prior to formalin injection.

**Immunohistochemistry and Image Analysis** TNC immunohistochemistry and analysis was performed as previously described. Two hours after the formalin injection into the upper lip, animals were deeply anesthetized with 5% isoflurane and perfused transcardially through the ascending aorta with 0.1 M phosphate-buffered saline (PBS, 50 mL pH 7.4), followed by 10% neutral buffer formalin (100 mL, Sigma, St. Louis, MO, U.S.A.). Fos is a nuclear phosphoprotein product of the c-fos proto-oncogene and its expression in neuronal cells can be induced by various factors, including nerve growth factor, several second messengers and cell membrane events. Although Fos mRNA accumulates and reaches peak values only at 30–45 min post-stimulation, the synthesis of Fos protein takes approximately an hour and reaches peak values at 2 h following stimulation. In this regard, we selected the 2 h time point as the ideal time to evaluate Fos expression following orofacial formalin injection. The tissues obtained from mice sacrificed for Fos immunostaining were also used in pERK and pp38 immunohistochemistry. After perfusing, the brainstem was removed immediately and stored overnight at 4°C in the same fixative and then placed in a cryoprotectant solution (30% sucrose in PBS) for at least two nights at 4°C before sectioning. Serial transverse sections (30 µm) were cut from TNC using a cryostat (Leica Microsystems, Wetzlar, Germany) and collected in PBS. After preblocking with 5% normal goat serum plus 0.3% Triton X-100 in PBS at room temperature for one hour, free-floating TNC sections were then incubated overnight at 4°C with primary antibodies against Fos (1:10000, Calbiochem, EMD Biosciences, San Diego, CA, U.S.A.), phospho-ERK (pERK, 1:1000, Cell Signaling, Danvers, MA, U.S.A.) or phospho-pp38 (pp38, 1:1000, Cell Signaling, Danvers, MA, U.S.A.) in diluents (1% normal donkey serum plus 0.3% Triton X-100 in PBS) at 4°C for overnight. Following several washes with PBS, the tissue sections were incubated with Cy3-conjugated secondary antibodies (1:500, Jackson ImmunoResearch, West Grove, PA, U.S.A.) for 4 h at room temperature. After several washes with PBS, the tissue sections were mounted onto slides under a cover slip.

TNC tissues were scanned with ECLIPSE 80i (Nikon Corp., Kanagawa, Japan) fluorescent microscope, and images of individual sections were digitized with 4096 grayscale values using a cooled CCD camera (CoolSnap ES model, Nihon Roper, Tokyo, Japan). The number of Fos-, pERK- or pp38-ir cells was counted using a computer-assisted image analysis system (Metamorph, Universal Imaging Co., Westchester, PA, U.S.A.). At the time of quantitation, the investigator performing the counting was blinded to the treatment.

**Statistical Analysis** All values are expressed as means ± S.E.M. Data analysis and statistical comparisons were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, U.S.A.). Comparison between two groups for immunohistochemistry experiment was performed using the Student’s t-test. For multiple comparisons in formalin pain behavior test, two-way repeated measures ANOVA followed by Bonferroni test or one-way ANOVA followed by post-hoc Newman–Keuls test was performed respectively. A p < 0.05 was considered statistically significant.

**RESULTS**

**Effect of BD1047 in Orofacial Formalin-Induced Pain Responses** Mice that received i.p. saline and orofacial formalin injection exhibited typical biphasic pain behaviors during the 45 min observation period (first phase: 0–6 after formalin injection and second phase: 6–45 min after formalin injection). Time course nociceptive responses in BD1047-treated groups (3 or 10 mg/kg) were significantly decreased at 8th block (21–24 min) after formalin injection (Fig. 1A, \( *p < 0.05, ***p < 0.001 \) as compared to those in saline-treated group; group factor: \( F(3, 270) =5.357, p=0.0142 \); time factor: \( F(15, 270)=22.290, p<0.0001 \)). The sum of orofacial formalin-induced rubbing behavior time in saline-treated group was 76.1 ± 14.3 s during the first phase and 397 ± 42.2 s during second phase. Pretreatment with Sig-1R antagonist BD1047 (10 mg/kg, \( n=6 \)) significantly inhibited formalin-induced pain behavior during both the first and the second phase of the formalin test, and the rubbing time was 41.1 ± 11.8 s and 198.4 ± 57.3 s during the first and the second phase, respectively. (Fig. 1B, \( *p < 0.05 \) as compared to those in saline-treated mice, \( n=6 \); the first phase: \( F(3, 18)=4.611, p=0.0228 \); the second phase: \( F(3, 18)=4.234, p=0.0294 \)).

**Effect of BD1047 in Fos Expression in TNC** The effect of i.p. pretreatment of BD1047 (10 mg/kg) in Fos expression in ipsilateral TNC region was shown in Fig. 2. Pretreatment of BD1047 (10 mg/kg, \( n=6 \), Figs. 2C, D) significantly reduced the formalin-induced increase in Fos expression in the ipsilateral TNC (Figs. 2A, B, E; \( *p < 0.05 \) as compared to those in saline-treated mice, \( n=6 \); t=3.131). Arrows in Figs. 2B, D show representative Fos-ir cells in the saline-treated mice and the BD1047-treated mice, respectively (magnified images of rectangle in Figs. 2A, C).

**Effect of BD1047 in pERK and pp38 MAPK Expression in TNC** The effect of i.p. pretreatment of BD1047 (10 mg/kg) in pERK and pp38 MAPK expression in ipsilateral TNC region was shown in Fig. 3. The number of pERK-ir cells in the TNC region in BD1047-treated group (Figs. 3B, E, \( n=6 \)) was not changed as compared to that in saline-treated group (Figs. 3A, E, \( n=6 \); t=0.414, the arrows indicate the representa-
tive pERK-ir cells). On the other hand, the number of pp38-ir cells in the ipsilateral TNC region was significantly reduced in BD1047-treated mice (Figs. 3D, F, n = 6) as compared to those in saline-treated mice (Figs. 3C, F, n = 6; *p < 0.05; t = 3.086). The arrows in Figs. 3C, D also show the representative pp38-ir cells.

DISCUSSION

The present study demonstrated that i.p. pretreatment of BD1047 (10 mg/kg) produced significant anti-nociceptive effects in mice orofacial formalin test. In addition, we examined that the number of Fos-ir cells in ipsilateral TNC was also reduced in BD1047-treated group as compared to that in saline-treated mice (Figs. 3C, F, n = 6; *p < 0.05; t = 3.086). The arrows in Figs. 3C, D also show the representative pp38-ir cells.

The ligand, BD1047, shows a high affinity for Sig-1R (Kᵢ = 0.93 nM) without other receptor interaction and thus is commonly used to evaluate the specific role of the Sig-1R. BD1047 can cross readily the blood–brain barrier in CNS, and thus systemically injected BD1047 has been widely used for antagonizing the CNS Sig-1Rs in lots of literatures related to learning and memory impairment, depression, anxiety or pain. Although BD1047 itself has no effect in normal condition of animals, it showed modulatory effects via Sig-1Rs under the pathophysiological condition in CNS. In this regard, Kwon et al. reported that capsaicin-induced TNC neuronal activity (i.e., increase of Fos-ir cells) was dose-dependently blocked by intracisternal BD1047, indicating that Sig-1R antagonists may be effective in the treatment of migraines.

In general, the amount of Fos-ir cells in the TNC has a strong positive correlation with the intensity of nociceptive stimulation. More importantly, we demonstrated that
orofacial formalin-induced Fos expression in the TNC was also decreased with BD1047 treatment. Therefore, it is possible that the specific activation of Sig-1R is closely associated with the pathophysiology of orofacial pain processing.

The plantar formalin test is commonly well established in both rats and mice as a reliable means of assessing analgesic effects and interactions between analgesic effects. Thus, the orofacial formalin test also represents a useful animal model of acute inflammatory nociception in the trigeminal nerve innervated region.1) The typical time course of the response to formalin stimulus is biphasic, with an early and short-lasting first phase followed, after a quiescent period, by a second prolonged phase.39) In the first phase, the neurogenic pain is caused by direct activation of C fibers nociceptive nerve endings,17) while the second phase is characterized as inflammatory pain, related to the release of chemical mediators such as histamine, serotonin, bradykinin, prostaglandins and excitatory amino acids, which can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) and central analgesics.40,41) We have previously determined that intrathecal injection of BD1047 reduced nociceptive responses in the second phase, but not the first phase of plantar formalin test.10) In contrast, Sig-1R knockout mice showed the anti-nociception in both first and second phase in mice formalin test.42) Moreover, the subcutaneous injection of haloperidol, which is known to be a Sig-1R antagonist, or its metabolites (reduced or oxidative form of haloperidol) suppressed the nociceptive responses in both phases.43) In this study, we also observed that i.p. injection of BD1047 reduced the nociceptive behaviors in both the first and the second phases of the orofacial formalin test in mice. These findings indicate that systemic inhibition of Sig-1R can block pain signaling in both the first and the second phases in plantar or orofacial formalin test, while spinal blockade of Sig-1R is only involved in the anti-nociceptive effect in the second phase pain response.

A large number of studies have provided evidences that MAPK pathways contribute to pain sensitization after tissue and nerve injury via distinct molecular and cellular mechanisms. Activation (e.g. phosphorylation) of MAPK under different persistent pain conditions results in the induction and maintenance of pain hypersensitivity via non-transcriptional and transcriptional regulation.44) In particular, phosphorylated ERK (pERK) and p38 (pp38) MAPK in the spinal cord dorsal horn cells plays an important role in the induction and maintenance of pain hypersensitivity caused by partial sciatic nerve injury.9,44–46) Recently, Son and Kwon revealed that both allodynia induction and spinal pERK elevation were completely prevented during the time of BD1047 treatment (0–5 d after chronic compression on the DRG).47) Furthermore, the Sig-1R knockout mice did not show any increase of pERK in the spinal cord after sciatic nerve injury,9) suggesting that Sig-1Rs might be related to the mechanism of induction of ERK activation in the neuropathic condition. However, the present data showed that BD1047 did not modify the expression of pERK in orofacial formalin-induced pain signaling. This discrepancy observed between our result and those of other studies seems to be due to the difference between the regions of pain signal transmission (spinal dorsal horn vs. TNC) or the animal models according to the type of pain condition (acute inflammatory pain vs. chronic neuropathic pain).

Fig. 2. Effect of BD1047 (10 mg/kg) on the Number of Fos-Immunoreactive (ir) Cells in the Ipsilateral TNC 2 h after Orofacial Formalin Injection

Photomicrographs of representative TNC sections illustrating Fos-ir cells in saline-treated mice (A and B) and BD1047-treated mice (C and D). B and D are magnified images of rectangle in panels A and C, respectively. Treatment of BD1047 significantly reduced the increase in Fos expression in the ipsilateral TNC (E). **p<0.01 vs. saline. Arrows show representative Fos-ir cells. Scale bar=200 µm.
On the other hand, we found that the number of pp38-ir cells can be reduced in BD1047-treated mice as compared to that in saline treated group. It has been reported that p38 MAPK plays an important role in a variety of chronic pain states.45,46,48) Especially, Li et al. showed that the expression of c-Fos and pp38 MAPK in the spinal cord dorsal horn were increased after an injection of formalin into the paw. 49) Recent study from Tan et al. also reported that the activation of the Src/p38 MAPK signaling cascade in spinal microglia contributed to late stage persistent mechanical hyperalgesia evoked by formalin injection into the paw. 50) In addition, our previous study revealed that the increase in spinal pp38 MAPK is closely associated with the induction of Sig-1R mediated mechanical allodynia in normal mice and neuropathic pain rats.30 Because p38 MAPK activation is regulated by elevated concentrations of intracellular Ca$^{2+}$ and the activation of Ca$^{2+}$ dependent enzymes,52,53) and Sig-1R sense endoplasmic reticulum Ca$^{2+}$ concentration,12,54) it is reasonable that changes in p38 MAPK phosphorylation in TNC might be regulated by Sig-1Rs, which may ultimately contribute to orofacial formalin-induced pain signaling.

In conclusion, the present study demonstrated that BD1047 significantly reduced nociceptive responses in both the first and the second phases in orofacial formalin test. The numbers of For-ir cells and pp38-ir cells in ipsilateral side of TNC were also reduced by BD1047 as compared to those in saline-treated animals, while the number of pERK-ir cells was not modified by BD1047. Taken together, these findings suggest that Sig-1Rs play a pivotal role in the orofacial pain processing, and the pp38 signaling pathway in TNC may be involved in the effect of BD1047.

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Fig. 3. Effect of BD1047 (10 mg/kg) on the Number of pERK (A, B and E)- and pp38 (C, D and F)-Immunoreactive (ir) Cells in the Ipsilateral TNC 2 h after Orofacial Formalin Injection

Photomicrographs of representative TNC sections illustrating pERK-ir (A and B) and pp38-ir cells (C and D) in saline-treated mice (A and C) and BD1047-treated mice (B and D), respectively. The number of pp38-ir cells in ipsilateral TNC was significantly decreased in BD1047-treated animals (F), while the number of pERK-ir cells was not changed (E). *p<0.05 vs. saline. Arrows show representative pERK and pp38-ir cells, respectively. Scale bar=100 µm.
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