Clonidine Reduces Nociceptive Responses in Mouse Orofacial Formalin Model: Potentiation by Sigma-1 Receptor Antagonist BD1047 without Impaired Motor Coordination

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Although the administration of clonidine, an alpha-2 adrenoceptor agonist, significantly attenuates nociception and hyperalgesia in several pain models, clinical trials of clonidine are limited by its side effects such as drowsiness, hypotension and sedation. Recently, we determined that the sigma-1 receptor antagonist BD1047 dose-dependently reduced nociceptive responses in a mouse orofacial formalin model. Here we examined whether intraperitoneal injection of clonidine suppressed the nociceptive responses in the orofacial formalin test, and whether co-administration with BD1047 enhances lower-dose clonidine-induced anti-nociceptive effects without the disruption of motor coordination and blood pressure. Formalin (5%, 10 µL) was subcutaneously injected into the right upper lip, and the rubbing responses with the ipsilateral fore- or hind-paw were counted for 45 min. Clonidine (10, 30 or 100 µg/kg) was intraperitoneally administered 30 min before formalin injection. Clonidine alone dose-dependently reduced nociceptive responses in both the first and second phases. Co-localization for alpha-2A adrenoceptors and sigma-1 receptors was determined in trigeminal ganglion cells. Interestingly, the sub-effective dose of BD1047 (3 mg/kg) significantly potentiated the anti-nociceptive effect of lower-dose clonidine (10 or 30 µg/kg) in the second phase. In particular, the middle dose of clonidine (30 µg/kg) in combination with BD1047 produced an anti-nociceptive effect similar to that of the high-dose clonidine, but without a significant motor dysfunction or hypotension. In contrast, mice treated with the high dose of clonidine developed severe impairment in motor coordination and blood pressure. These data suggest that a combination of low-dose clonidine with BD1047 may be a novel and safe therapeutic strategy for orofacial pain management.

Key words alpha-2 adrenoceptor; clonidine; sigma-1 receptor; orofacial pain; formalin test
the orofacial pain.

While clonidine can produce a predominantly inhibitory modulation of the N-methyl-D-aspartate (NMDA)-evoked responses of nociceptive neurons in the medullary dorsal horn, the activation of Sig-1Rs is also closely associated with the NMDA-mediated nociceptive behaviors via the phosphorylation of NMDA receptors in spinal dorsal horn. Thus, it is possible that the modulation of Ca^{2+} signaling by NMDA receptors may be a critical direct or indirect mechanism to the pharmacological action of clonidine or Sig-1R antagonist, BD1047 in orofacial pain condition.

In this regard, the present study was designed to verify whether intraperitoneal injection of clonidine, an alpha-2 adrenoceptor agonist, suppresses the nociceptive responses in orofacial formalin mouse model, and whether alpha-2 adrenoceptors are co-localized with Sig-1Rs in trigeminal ganglion (TG), and whether co-administration with BD1047 enhances lower-dose clonidine-induced anti-nociceptive effects, and finally determined whether these treatments affect the motor coordination and the blood pressure.

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).

Drugs Clonidine (Sigma, St. Louis, MO, U.S.A.) or Sig-1R receptor antagonist, BD1047 (Tocris, Bristol, U.K.) were diluted in physiological saline. Clonidine at a dose of 10, 30 or 100 µg/kg was intraperitoneally (i.p.) injected 30 min prior to formalin injection. The doses of clonidine was chosen based on the previous studies.

For combination treatment test, BD1047 (3 mg/kg) was co-administrated with each dose of clonidine.

Formalin-Induced Orofacial Pain Test Formalin was prepared from commercially available stock formalin (aqueous solution of 37% formaldehyde, Sigma) 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 hind-paw. 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.

Rota-Rod Test The rota-rod test is a commonly used screening procedure to detect motor incoordination and/or ataxia in rodents. We have also used the rota-rod test for mice to examine the potential sedative effects of clonidine.

The Roto-rod apparatus (model# DJ-4009; Dae-Jong Engineering & Clean Technology, Seoul, Korea) consisted of a rotating horizontal bar (diameter=6 cm), which was subdivided into 4 compartments by rotating plates. All mice were placed on the horizontal bar, which was set at a rotation speed of 4 revolutions per minutes. Twenty-four hours before the actual rota-rod test all mice were tested and those that were able to remain on the rod for at least 120 s were included in the study.

Thirty minutes after clonidine injection, each animal was subsequently tested on the rota-rod over a 2-min period and their performance time on the bar (in s) was measured. The test was repeated 3 times and the mean value for each animal was recorded.

Evaluation of Blood Pressure Systolic blood pressure (SBP) was also measured using a noninvasive computerized tail-cuff system (PowerLab system; ADI Instrument Pry Ltd., Chain Hills, NSW, Australia) as previously described. Briefly, animals were acclimated for 1 h in a quiet test room before obtaining cardiovascular measurements. At the end of the 1-h period, the SBP was measured. This experiment was repeated 3 times and the mean value for each animal was recorded. Blood pressure was recorded 5 min (PRE) prior and 30 min (POST) after treatment of clonidine or BD1047 and their co-administration.

Immunohistochemistry Immunohistochemistry was performed as previously described. 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). After perfusing, the TG 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 sections (15 µm) were cut from TG using a cryostat (Leica Microsystems, Wetzlar, Germany). The thaw-mounted TG tissues were preblocked with 5% normal donkey serum plus 0.3% Triton X-100 in PBS at room temperature for one hour. Double-immunofluorescence staining was used to study the distribution of alpha-2A adrenoceptors and Sig-1Rs in TG cells. TG sections were incubated at 4°C for 48 h with goat alpha-2A adrenoceptor antibody (1: 500; Abcam Inc., Cambridge, MA, U.S.A.) in combination with rabbit Sig-1R antibody (1: 500; Abcam Inc.). After washing with PBS, the sections were also reacted at 4°C for 48 h in a dark chamber with the following combination of secondary antibodies; Alexa fluor 488-conjugated anti-goat immunoglobulin G (IgG) (1: 500; Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) and Cy3-conjugated anti-rabbit IgG (1: 500; Jackson ImmunoResearch Laboratories). Possible co-localization of alpha-2A receptors with Sig-1R in TG sections was visualized using a fluorescent microscope (ECLIPSE 80i, Nikon Corp., Kanaga-
Statistical Analysis

Sample size was estimated by power analysis using G* power 3.1 (Faul, University of Kiel, Kiel, Germany) with power=0.8 and alpha=0.05 based on the data from our previous studies (5–7 animals depending on animal availability). All values are expressed as mean±standard error of the mean (S.E.M.). Data analysis and statistical comparisons were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, U.S.A.). For multiple comparison test, two-way repeated measures ANOVA or one-way ANOVA followed by a post hoc Bonferroni test was used, respectively. A p<0.05 was considered statistically significant.

RESULTS

Dose-Dependent Effect of Clonidine 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 clonidine-treated mice (30 or 100 µg/kg) were significantly decreased 12–24 min after formalin injection (*p<0.05, **p<0.01 vs. saline). In sum data of nociceptive responses in the first or the second phase, clonidine administration significantly reduced the nociceptive responses in the first (B) or the second phase (C), respectively. *p<0.05, ***p<0.001 vs. saline.

Fig. 1. Effect of Clonidine (10, 30 or 100 µg/kg) on Orofacial Formalin-Induced Nociceptive Responses during Total Time Blocks (A) and Time Blocks Divided into the First and the Second Phase (B)

Time course nociceptive responses in clonidine-treated groups (30 or 100 µg/kg) were significantly decreased formalin induced nociception (A, *p<0.05, **p<0.01 as compared to those in saline-treated group, n=5–7 per group). The sum of orofacial formalin-induced rubbing behavior time in saline-treated group was 88.7±9.7 s during the first phase and 270.1±32.1 s during the second phase. Pretreatment with clonidine (30 and 100 µg/kg) at X200 and digitized using a cooled CCD camera (CoolSnap ES model, Nihon Roper, Tokyo, Japan).

Fig. 2. Colocalization of Alpha-2A Adrenoceptors (A) and Sigma-1 Receptors (B) in Trigeminal Ganglion

Merged image (C) revealed that most of Sig-1Rs positive cells (B) colocalized with alpha-2A adrenoceptors (A) in TG cells (the arrowheads indicate alpha-2A adrenoceptors-ir cells, and the arrows indicate representative cells colocalized with Sig-1Rs, scale bar=100µm).
kg) dose-dependently suppressed this sum of formalin-induced pain responses during both the first and the second phases. (Figs. 1B, C, *p<0.05, ***p<0.001 as compared to those in saline-treated mice, n=5–7 per group).

**Co-localization of Alpha-2A Adrenoceptors and Sigma-1 Receptors in Trigeminal Ganglion** Immunoreactivity for alpha-2A adrenoceptors (Fig. 2A) and Sig-1Rs (Fig. 2B) was observed in TG cells. Immunoreactivity for alpha-2A adrenoceptors was extensively found in TG cells, whereas Sig-1Rs were predominantly localized in small or medium sized cells of TG. Merged images revealed that most of Sig-1Rs positive cells colocalized with alpha-2A adrenoceptors in TG (Fig. 2C, the arrowheads indicate alpha-2A adrenoceptors-ir cells, and the arrows indicate representative cells colocalized with Sig-1Rs).

**Effect of Co-administration of Clonidine with Sig-1R Antagonist, BD1047 in Orofacial Formalin-Induced Pain Responses** In Fig. 1, intraperitoneal clonidine alone injection produced a dose-dependent anti-nociceptive effect on orofacial formalin-induced nociceptive responses during both the first and the second phases. By contrast, injection of sub-effective dose of BD1047 alone (3 mg/kg, n=6) did not produce a de-

![Graph](image)

**Fig. 3. Effect of Co-administration of Clonidine with Sig-1R Antagonist, BD1047 in Orofacial Formalin-Induced Pain Responses**

The injection of sub-effective dose of BD1047 (BD, 3 mg/kg) did not decrease orofacial formalin-induced nociceptive responses during the first or the second phase (A). Co-administration of clonidine with BD1047 (3 mg/kg) produced more potent anti-nociceptive effect during the second phase (C), but not the first phase (B), as compared to those in clonidine alone-injected mice (*p<0.05, **p<0.01, ***p<0.001 as compared to those in saline-treated mice, and ## p<0.01 as compared to those in clonidine alone (10 or 30 mg/kg)-treated mice).
crease in orofacial formalin-induced nociceptive responses during the first or the second phase (Figs. 3A–C). Interestingly, co-administration of clonidine with BD1047 (3 mg/kg) caused more potent anti-nociceptive effect during the second phase, but not the first phase, as compared to those in 10 or 30 mg/kg clonidine alone-injected mice (Figs. 3A–C, *p < 0.05, **p < 0.01, ***p < 0.001 as compared to those in saline-treated mice, and ##p < 0.01 as compared to those in clonidine alone-treated mice, n=6 per group).

Effect of Clonidine Alone and Co-administration with BD1047 on Motor Performance and Blood Pressure In the rota-rod test, saline or BD1047 (3 mg/kg) injection did not affect motor coordination (Figs. 4A, B, n=6 per group). Intraperitoneal injection of high dose of clonidine (100 µg/kg) significantly decreased performance time in rota-rod test (***p<0.001 as compared to those in saline-treated mice), whereas neither 10, 30 mg/kg clonidine nor their combination with BD1047 (3 mg/kg) affected the motor function (n=6 per group).

In the blood pressure test, saline or BD1047 (3 mg/kg) injection did not affect normal blood pressure (Figs. 4C, D). On the other hand, the highest dose of clonidine (100 µg/kg) caused a significant decrease in blood pressure (Fig. 4C, ***p<0.001 as compared to those in saline-treated mice, n=6 per group). In contrast, lower doses (10 and 30 µg/kg) of clonidine alone or when co-administered with BD1047 (3 mg/kg) did not produce hypotension (Figs. 4C, D, n=6 per group).

DISCUSSION

The present study demonstrated that i.p. administration of clonidine dose-dependently produced anti-nociceptive effects during both the first and second phases in mice orofacial formalin test. Activation of alpha-2 adrenoceptor is well known to play an important role in mediating anti-nociceptive effects. Especially, clonidine, an alpha-2 adrenoceptor agonist, has been reported to produce potent anti-nociception in animals and human studies. More recently, we also examined that intrathecal clonidine significantly reduced mechanical allodynia and thermal hyperalgesia in rat neuropathic pain model and nociceptive responses in mice formalin test. By contrast to those literatures, there has been unclear whether several types of orofacial pain can be relieved by treatment of clonidine. Cahusac et al. reported that selective descending inhibition of nociceptive responses in neurons of the rat caudal trigeminal nucleus is mediated by norepinephrine, possibly by an action at alpha-2 adrenoceptors. It was also verified that clonidine produces a predominantly inhibitory modulation of the NMDA-evoked responses of nociceptive neurons in the medullary dorsal horn. Moreover, Wang
et al. examined functional role of alpha-2 adrenoceptor subtypes in modulating the NMDA-induced nociceptive behavior in the medullary dorsal horn by using antisense oligodeoxynucleotides to selectively knock-down the receptor subtypes. They revealed that the alpha-2A adrenoceptor rather than alpha-2C adrenoceptor, mainly mediated the anti-nociceptive effect of clonidine in the medullary dorsal horn in the rat. In this regard, we first showed that clonidine could reduce acute inflammatory pain responses in orofacial regions, and this anti-nociceptive effect of clonidine might be associated with activation of alpha-2A adrenoceptor in the trigeminal innervated regions.

On the other hand, Sig-1Rs have been also well recognized to play an important role in a variety of cellular functions via modulation of intracellular Ca\(^{2+}\) concentration. Our previous studies have shown that pharmacological blockade of Sig-1Rs using BD1047 treatment suppressed nociceptive signaling under acute or chronic pain condition using the hind paw formalin-induced pain test or the sciatic nerve injury-induced pain animal models. In addition, we have recently reported that i.p. treatment of BD1047 dose-dependently reduced orofacial nociceptive responses and the expression of c-Fos protein, a nociceptive marker in the TNC in the mouse orofacial formalin model. Based on these findings, we were to examine whether co-administration of clonidine with BD1047 enhances clonidine-induced anti-nociceptive effects. Firstly we determined that alpha-2A adrenoceptors were co-localized with Sig-1Rs in TG neurons. Immunoreactivity for alpha-2A adrenoceptors was extensively found in TG cells, while Sig-1Rs were predominantly localized in small or medium sized cells. Merged images revealed that most of Sig-1Rs-ir cells colocalized with alpha-2A adrenoceptors. The Sig-1Rs are widely distributed in mammalian central nervous system including certain cortical areas, the hypothalamus and the dorsal horn of the spinal cord. In addition, we reported that Sig-1Rs in the spinal dorsal horn are co-localized with astrocytes, which is involved in the development of mechanical allodynia in chronic constriction nerve injured mice. However, it is also well-known that Sig-1Rs are localized in the peripheral tissues including nociceptive nerve endings. Recently, Bangaru et al. reported that Sig-1Rs are present in both sensory neurons and satellite cells in rat DRGs, which are down-regulated after peripheral nerve injury. These findings demonstrate that Sig-1Rs can be differentially located in the spinal cord and DRG (or TG). Secondly we verified that the sub-effective dose of BD1047 (3 mg/kg) significantly potentiated the anti-nociceptive effect of lower-dose clonidine (10 or 30 \(\mu\)g/kg) during the second phase, although BD1047 (3 mg/kg) alone did not produce an anti-nociceptive effect during the first or second phases. These findings demonstrated that co-administration of clonidine with BD1047 could produce more potent anti-nociceptive effect during the second phase as compared to that of clonidine alone.

An alpha-2 adrenoceptor is coupled to a Gi protein, which reduces the activity of adenylyl cyclase, simultaneously suppressing both the production of cAMP and the activity of PKA. This general mechanism may be mainly associated with the anti-nociceptive effect of clonidine in orofacial pain. However, clonidine treatment is also thought to produce analgesia in spinal cord level both by reducing the release of glutamate and substance P from central afferent terminals and by hyperpolarizing dorsal horn neurons. These phenomena are related to the inhibition of N-type Ca\(^{2+}\) channels on the presynaptic membrane and to an increase in the conductance of inwardly rectifying potassium channels, respectively. This modulation of Ca\(^{2+}\) signaling by clonidine may be also closely related to the action of Sig-1Rs in TG or TNC regions. In this regard, Sig-1Rs are abundantly located in the plasma membrane and subcellular membranes, particularly in the endoplasmic reticulum, in which they play a modulatory role in intracellular Ca\(^{2+}\) signaling. Although the endogenous ligands for Sig-1R have not yet been defined unequivocally, currently various neurosteroids such as pregnenolone, dehydroepiandrosterone (DHEA), their sulfate esters, progesterone and allopregnenolone are known to interact with Sig-1Rs. Recently we have reported that the activation (e.g. phosphorylation) of intracellular p38 mitogen-activated protein kinases (MAPK) mediates the action of Sig-1R, which ultimately leads to the mechanical allodynia, but not thermal hyperalgesia in sciatic nerve injured mice. In addition, we showed that the anti-nociceptive effect of BD1047 in orofacial region was also associated with the suppression of phosphorylated p38 MAPK, but not extracellular signal-regulated kinase (ERK) pathway. Thus it is possible that the modulation of intracellular nociceptive signaling (e.g. Ca\(^{2+}\) signaling or p38 MAPK) by sub-effective dose of BD1047 can affect anti-nociceptive effect induced by lower-dose clonidine in orofacial formalin induced pain responses.

Finally we examined whether several dose of clonidine alone or co-administration with BD1047 affect the motor coordination and blood pressure. Intraperitoneal injection of high dose of clonidine (100 \(\mu\)g/kg) significantly decreased performance time in rota-rod test, and induced hypotension. In contrast, neither 10, 30 mg/kg clonidine nor their combination with BD1047 (3 mg/kg) affected the motor function and blood pressure. It is well known that the maximal effective dose of clonidine is typically accompanied by significant side effects that include hypotension, bradycardia, lethargy and weakness, which serve to limit the use of clonidine particularly at higher, clinically effective doses. The present study showed that BD1047 treatment significantly potentiated the clonidine induced anti-nociceptive effect during the second phase of the formalin test. Importantly, pairing low-dose clonidine treatment with BD1047 did not induce any changes in motor and vascular effect, suggesting the use of this co-administration of clonidine with BD1047 to treat orofacial pain without the unwanted side effects of typical higher doses of clonidine.

In conclusion, the current study demonstrated that clonidine dose-dependently reduced nociceptive responses in orofacial formalin induced pain model. In addition, co-administration of clonidine with BD1047 consistently potentiated clonidine alone-induced anti-nociception. The highest dose of clonidine (maximal anti-nociceptive dose, 100 \(\mu\)g/kg) was associated with the impairment of motor coordination and blood pressure. However, when lower dose of clonidine (30 \(\mu\)g/kg) were combined with BD1047, the anti-nociceptive effect obtained was equivalent to that of the high dose of clonidine but without an impaired motor coordination and hypotension. Taken together, these findings imply that the combined therapeutic approach of low-dose clonidine with BD1047 decreases the side effects of drug therapy and suggest a possibility of novel strategy for orofacial pain management.
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Conflict of Interest The authors declare no conflict of interest.

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