Effects of Topical Application of Clonidine Cream on Pain Behaviors and Spinal Fos Protein Expression in Rat Models of Neuropathic Pain, Postoperative Pain, and Inflammatory Pain

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Background: Clonidine can effectively reduce pain and/or hypersensitivity. However, the antihypersensitivity effects of clonidine topically applied in cream (CC) have not been investigated. The authors evaluated effects of topical application of CC on pain behaviors and spinal Fos-like immunoreactivity in rats with hypersensitivity.

Methods: Clonidine (30, 100, and 300 μg/g) was prepared in a cream base. In rat models of neuropathic pain, inflammatory pain, and postoperative pain, the authors evaluated effects of CC (6.1 g), topically applied onto the plantar surface of the injured or uninjured paw, on thermal hyperalgesia and mechanical allodynia to von Frey filaments. The authors also evaluated effects of CC on lumbar spinal Fos-like immunoreactivity.

Results: In neuropathic rats, CC applied onto the injured paw reduced thermal hyperalgesia and mechanical allodynia dose dependently, whereas CC applied onto the uninjured paw had no effect. The antihypersensitivity effects of CC were antagonized by intraperitoneal yohimbine (10 mg/kg). Further, CC reduced Fos-like immunoreactivity in neuropathic rats. In contrast, CC in a single dose had no effects on hyperalgesia, allodynia, or Fos-like immunoreactivity in rats with inflammatory pain or postoperative pain. In rats with postoperative pain, CC repeatedly applied for 6 days reduced thermal hyperalgesia, but not mechanical allodynia, in the postoperative days, whereas it had no effects on hyperalgesia or allodynia in those with inflammatory pain.

Conclusions: Topical CC in concentrations examined significantly reduced hypersensitivity and lumbar spinal Fos-like immunoreactivity in rats with neuropathic pain, probably through activation of peripherally located α2 adrenoceptors. However, CC was only partially effective and totally ineffective in rats with postoperative pain and inflammatory pain, respectively.

CLONIDINE, an α2-adrenergic agonist, is an extremely potent analgesic agent.1 However, adverse effects, such as sedation and hypotension, limit its clinical use.2 Given these undesirable centrally mediated side effects, it may be advantageous to apply clonidine topically, to the site of pain origin. With topical treatment, one may achieve analgesic efficacy due to high drug concentration at the site of pain origin while avoiding high blood drug concentration and thus centrally mediated side effects.3,4 Because α2 adrenoceptors are located not only in the central nervous system but also on dorsal root ganglion (DRG) cells,5,6 topical clonidine may produce antinociception and/or antihypersensitivity. Several previous studies have shown the antinociception/antihypersensitivity from peripherally administrated clonidine, including topical clonidine given via tail immersion in an animal model of nociceptive pain,7 intraarticular clonidine in an animal inflammatory pain model,7 periarticular clonidine in an animal neuropathic pain model,8,9 intraarticular clonidine in humans undergoing knee arthroscopy,10 and topical clonidine delivered via a patch in patients with sympathetically maintained pain.11 To date, however, the antinociceptive and/or antihypersensitivity effects of clonidine topically given in cream has not been studied in animals or humans, except one pilot study in patients with oral neuropathic pain or neuralgia.12 A previous study has shown that intrathecal clonidine reduces allodynia in a rat model of postoperative pain.13 Further, several studies have suggested that α2-adrenoceptor agonists have increased analgesic efficacy or potency against hypersensitivity states induced by inflammation and nerve injury than against acute nociceptive pain.14-17 Therefore, α2-adrenoceptor agonists may be effective in relieving hypersensitivity states associated with neuropathic pain, postoperative pain, and inflammatory pain. Currently, however, no systematic data are available regarding the antihypersensitivity effects of clonidine cream in these hypersensitivity states. In addition, to our knowledge, no study has been conducted to compare the antihypersensitivity effects of topical clonidine among these pathophysiologic pain conditions. The current comparative study was designed to determine whether clonidine cream can reduce hypersensitivity in the rat models of neuropathic pain, postoperative pain, and inflammatory pain.
Materials and Methods

Animals
After approval from the Institutional Animal Care and Use Committee, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, male Sprague-Dawley rats weighing 250–300 g were studied. The animals were housed at 22°C and under a 12 h–12 h light–dark cycle, with free access to food and water.

Drugs
By courtesy of the University Hospital Pharmacy, clonidine (30, 100, and 300 μg/g) was prepared in a cream base, by mixing clonidine hydrochloride (Sigma Chemical, St. Louis, MO) with vehicle (Plastibase consisting of 5% polyethylene resin and 95% liquid paraffin). Yohimbine hydrochloride (1 and 10 mg/kg; Sigma Chemical) was dissolved in normal saline (0.3 ml) for intraperitoneal injection. λ-Carrageenan (Sigma Chemical) was suspended in normal saline by sonication to make 2% solution.

Neuropathic Pain Model
Spinal nerve ligation (SNL), i.e., tight ligation of the left L5 and L6 spinal nerves with 6-0 silk sutures, was performed under microscope in halothane-anesthetized rats, as previously described. Animals were allowed to recover until the seventh postoperative day, when drug tests were performed.

Postoperative Pain Model
Incisional pain was introduced, as previously described. In halothane-anesthetized rats, a 1-cm longitudinal incision was made through skin and fascia of the plantar hind foot, starting 0.5 cm from the proximal edge of the heel and extending toward the toes. The plantaris muscle was elevated, stretched, and incised longitudinally. After hemostasis with gentle pressure, the skin was closed. Postoperatively, animals were allowed to recover from anesthesia.

Inflammatory Pain Model
To induce local inflammation in halothane-anesthetized rats, 2% carrageenan (0.1 ml) was injected subcutaneously into the left plantar hind foot via a 27-gauge needle, as previously described. After carrageenan injection, animals were allowed to recover from anesthesia.

Evaluation of Thermal Hyperalgesia
Thermal hyperalgesia was assessed using a thermal testing apparatus (7370; Ugo Basile, Comerio, Italy), as previously described. Briefly, rats were placed in plastic boxes on a glass surface maintained at 30°C and were allowed to acclimate for 15 min. Latency to paw withdrawal in response to a radiant heat stimulus (paw withdrawal latency [PWL]) was determined using an intense light focused on the hind paw from underneath the glass pane. Animals were acclimated to the procedure until stable PWL values were obtained, and the mean PWL value of the last three measurements were used for analysis. Light intensity was precalibrated so that the baseline PWL was approximately 10 s. A cutoff time was set at 20 s to avoid tissue damage.

Evaluation of Mechanical Allodynia
Sensitivity to a mechanical stimulus was assessed with calibrated von Frey filaments (Stoelting, Wood Dale, IL), as previously described. Briefly, animals were placed in individual plastic boxes on a wire floor and allowed to acclimate for 15 min. Precalibrated von Frey filaments were applied perpendicularly to the plantar hind foot and pressed to the point of bending over 6 s. The filaments were applied in increasing order of bending force until a brisk withdrawal or paw flinching occurred. The mean paw withdrawal threshold (PWT) value obtained from three procedures, spaced at 2-min intervals, was used for analysis.

In the current study, we did not test for centrally mediated side effects of clonidine, such as sedation and motor dysfunction.

Immunobistochemical Study
Fos-like immunoreactivity in the lumbar spinal dorsal horn was quantified, as previously described. Briefly, rats anesthetized with intraperitoneal pentobarbital (100 mg/kg) were transcardially perfused with 100 ml phosphate buffer, 0.1 M, and then with 500 ml ice-cold 4% paraformaldehyde in phosphate buffer. The spinal cord was dissected and immersed in the same fixative for 24 h at 4°C, and then in 30% sucrose solution in phosphate buffer at 4°C overnight. The L4–L5 spinal cord was sectioned into 40-μm-thick sections. In consecutive order and at room temperature, sections were placed in phosphate-buffered solution; incubated for 1 h in blocking solution (5% normal rabbit serum and 0.3% Triton X in phosphate buffer); incubated overnight with goat anti-Fos antibody (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA) in 1% normal rabbit serum and 0.3% Triton X in phosphate buffer (buffer 1); incubated for 1 h in biotinylated rabbit anti-goat immunoglobulin (1:200; Chemicon, Temecula, CA) in buffer 1; vigorously rinsed with 0.3% Triton X in 0.1 M phosphate-buffered saline (buffer 2); and incubated for 1 h in avidin–biotin–peroxidase complex (Vectra Elite ABC; Vector Laboratories, Burlingame, CA) in buffer 2. Visualization of the reaction product was achieved by incubation for 4 min with dianinobenzidine and nickel–ammonium sulfate in the presence of hydrogen peroxide (dianinobenzidine kit; Vector Laboratories). After staining, the sections were placed on a glass
slide, dehydrated, cleared in 100% xylene, and coverslipped. Five sections were randomly selected in each rat, and rat-like immunoreactive neurons in the dorsal horn of the spinal cord ipsilateral to the injured paw were counted under the microscope.

**Topical Application of Clonidine Cream**

In unanesthetized rats, clonidine cream (0.1 g) of various concentrations (30, 100, and 300 µg/g) was applied onto the plantar surface of injured or uninjured hind paws. After application of clonidine cream, the animals were placed in plastic tunnels (6 cm diameter, 25 cm length) for 45 min, in which they could not move, escape, or turn around, to prevent licking of the injured hind paw and thus early removal of the cream. Animals were accustomed to this procedure for 3 days before topical clonidine application to minimize additional stress reactions during experiments.

**Single Topical Application of Clonidine Cream in the Neuropathic Pain Model**

In neuropathic rats, all drug tests were performed at 7 days after SNL. Four groups of animals received topical clonidine (3, 10, or 30 µg in 0.1 g clonidine cream) or vehicle (0.1 g), respectively, on the injured paw (n = 10 for each group). An additional two groups received clonidine (30 µg) on the injured paw, at 5 min after intraperitoneal injection of 1 or 10 mg/kg yohimbine, respectively (n = 6 for each group). Another two groups received topical clonidine (30 µg) or vehicle (0.1 g), respectively, on the uninjured paw (n = 6 for each group). PWL and PWT were measured in the bilateral hind paws before SNL; immediately before the topical drug application; and at 1, 2, 4, 6, 24, and 72 h after the topical drug application. For immunohistochemical staining, two groups of animals that did not receive thermal or mechanical stimuli for behavior testing were transcardially perfused with paraformaldehyde at 4 h after topical application of clonidine (30 g) or vehicle (0.1 g), respectively (n = 6 for each group).

**Repeated Topical Application of Clonidine Cream in the Postoperative Pain Model**

At 2 h after incision, four groups of animals received topical clonidine (3, 10, or 30 µg in 0.1 g clonidine cream) or vehicle (0.1 g), respectively, on the injured paw (n = 6 for each group). PWL and PWT were measured bilaterally before incision; immediately before the topical drug application; and at 1, 2, 4, 6, 24, and 72 h after the topical drug application. For immunohistochemical staining, two groups of animals that did not receive thermal or mechanical stimuli for behavior testing were transcardially perfused with paraformaldehyde at 4 h after topical application of clonidine (30 µg) or vehicle (0.1 g), respectively, onto the injured paw (n = 6 for each group).

**Statistical Analysis**

Results are presented as mean ± SEM, or median and percentiles, according to data types. Parametric data were analyzed with unpaired t tests, or analysis of variance, followed by Neumann-Keuls test for multiple comparisons. Nonparametric data were analyzed with Kruskal-Wallis test, followed by Dunnett test for multiple comparisons of nonparametric data. The criterion for statistical significance was set at P < 0.05.
Results

Single Topical Application of Clonidine Cream in the Neuropathic Pain Model

By 7 days after SNL, PWL and PWT decreased significantly in the injured paw, indicating the development of thermal hyperalgesia and mechanical allodynia, respectively (figs. 1A and B).

When applied onto the injured paw, clonidine cream resulted in significant and dose-dependent increases in PWL and PWT in the injured paw, compared with vehicle, indicating that clonidine cream reduced both thermal hyperalgesia and mechanical allodynia dose dependently (figs. 1A and B). These effects reached their maxima at 4 or 6 h after the topical clonidine application and disappeared within 24 h (figs. 1A and B). Clonidine cream applied onto the injured paw did not affect PWL or PWT in the uninjured paw (figs. 1C and D). Pretreatment with intraperitoneal yohimbine (10 mg/kg) antagonized both of the antihyperalgesic and antiallodynic effects of clonidine cream (figs. 2A and B).

When applied onto the uninjured paw, clonidine cream did not affect PWL or PWT in the injured (figs. 3A and B) or uninjured paw (data not shown), indicating that clonidine cream applied onto the uninjured paw did not produce any antihyperalgesic, antiallodynic, or antinociceptive effects in the injured or uninjured paw.

Typical examples of Fos-like immunoreactivity staining in the vehicle-treated and clonidine-treated groups are shown in figures 4A and B, respectively. In the neuropathic pain model, clonidine cream significantly reduced the number of Fos-like immunoreactive cells, compared with vehicle, in the dorsal horn ipsilateral to the injured paw (fig. 4C).

Single Topical Application of Clonidine Cream in the Postoperative and Inflammatory Pain Models

In the postoperative and inflammatory pain models, single topical application of clonidine cream onto the injured paw did not produce any significant effects on PWL or PWT, compared with vehicle, in the injured paw (fig. 5) or uninjured paw (data not shown), indicating that it did not produce any antihyperalgesic, antiallodynic, or antinociceptive effects. In these models,
clonidine cream did not decrease the number of Fos-like immunoreactive cells, compared with vehicle (fig. 4C).

Repeated Topical Application of Clonidine Cream in the Postoperative Pain Model

In the vehicle-treated group of the postoperative pain model, thermal hyperalgesia and mechanical allodynia reduced spontaneously and progressively as days elapsed since incision surgery. Compared with vehicle, clonidine cream did not reduce thermal hyperalgesia or mechanical allodynia at 2 or 4 h postoperatively on the day of surgery. However, topical clonidine repeatedly applied on the following days reduced thermal hyperalgesia significantly and dose dependently on the first to fourth postoperative days, when spontaneous alleviation of thermal hyperalgesia progressed (fig. 6A). In contrast, it did not reduce mechanical allodynia during the 6-day observation period despite spontaneous alleviation of allodynia (fig. 6B).

Repeating Topical Application of Clonidine Cream in the Inflammatory Pain Model

Clonidine cream repeatedly applied over 4 days did not reduce thermal hyperalgesia or mechanical allodynia, compared with vehicle, during the 4-day observation period despite spontaneous alleviation of hyperalgesia and allodynia during this period (figs. 6C and D).

Discussion

In the current study, we studied the effects of clonidine cream on thermal hyperalgesia and mechanical allodynia in the rat models of neuropathic pain, postoperative pain, and inflammatory pain, respectively. Furthermore, we investigated the effects of clonidine cream on spinal Fos-like immunoreactivity in these models. Fos, a protein product of the immediate early gene c-fos, is induced in the spinal dorsal horn after nociceptive stimulation.24,25 A number of studies have demonstrated that analgesics suppress Fos protein expression in parallel with suppression of pain behaviors,24,25 although a few reports describe dissociation between effects of analgesics on pain behaviors and Fos expression.27,28

Using these behavioral and immunohistochemical tests, we found that in rats with neuropathic pain, clonidine cream in a single dose could reduce thermal hyperalgesia and mechanical allodynia dose dependently, and decrease spinal Fos-like immunoreactivity. Clonidine cream, topically applied onto the injured paw,
had antiallodynic and antihyperalgesic effects in the injured paw exposed to clonidine, whereas it did not produce thermal or mechanical antinociception in the uninjured paw not exposed to the compound. Further, clonidine cream applied onto the uninjured paw did not exert any antiallodynic, antihyperalgesic, or antinociceptive effect in the injured or uninjured paw. These findings suggested that the antiallodynic and antihyperalgesic effects of clonidine cream observed in neuropathic rats were not due to this compound diffusing into the blood and acting at central sites, but due to direct action of this compound at the peripheral site.

The locally exerted antihypersensitivity effects of clonidine cream were blocked by an $\alpha_2$-adrenoceptor antagonist, yohimbine. Taken together, our data suggested that these effects of clonidine cream were mediated probably by $\alpha_2$ adrenoceptors expressed on primary afferent terminals. There are several lines of evidence that support this contention. First, $\alpha_2$ adrenoceptors are expressed on DRG neurons.5,6 Second, activation of $\alpha_2$ adrenoceptors inhibits the release of neurotransmitters, such as calcitonin gene–related peptide and glutamate, from DRG neurons.29,30 Third, clonidine at high concentrations produces a minor degree of nerve conduction blockade.31 Fourth, clonidine is a lipophilic substance which would likely penetrate the skin easily.32

Fig. 5. Effects of clonidine cream on the paw withdrawal latency (PWL; A) and the paw withdrawal threshold (PWT; B) in the postoperative pain model, and on PWL (C) and PWT (D) in the inflammatory pain model. PWL is shown as mean ± SEM, whereas PWT is shown as median and percentiles (n = 6 in each group). CA = carrageenan injection; CLO = clonidine in cream.

Fig. 6. Effects of repeated application of clonidine cream on the paw withdrawal latency (PWL; A) and the paw withdrawal threshold (PWT; B) in the postoperative pain model, and on PWL (C) and PWT (D) in the inflammatory pain model. PWL is shown as mean ± SEM, whereas PWT is shown as median and percentiles (n = 9 and 6 in each group of the postoperative and inflammatory pain models, respectively). *P < 0.05 versus vehicle. †P < 0.05 versus clonidine 3 μg. ‡P < 0.05 versus clonidine 30 μg. CA = carrageenan injection; CLO = clonidine in cream.
Last, several animal and human studies have actually demonstrated antinociceptive and/or antihypersensitivity effects of peripherally administered clonidine. Therefore, the consequences of peripherally located α₂-adrenoceptor activation may account for the antihypersensitivity from clonidine cream in neuropathic rats.

In the current study, clonidine cream at doses that did not produce antinociception produced antihypersensitivity effects in neuropathic rats. Likewise, a previous study showed that medetomidine, an α₂-adrenoceptor agonist, injected into the foot pad of the allodynic hind paw at a dose that did not produce antinociception effectively reduced mechanical allodynia in neuropathic rats. In that previous study, however, medetomidine reversed the unilateral allodynia independent of the site of administration (either the alldynic or the contralateral hind paw), and therefore, a central, rather than peripheral, α₂-adrenergic mechanism was considered to be involved in the antiallodynic action of medetomidine. The difference in the probable site of drug action (i.e., central vs. peripheral) between the previous and current studies may be related to differences in the drugs used (medetomidine vs. clonidine) or the route of drug administration (subcutaneous injection vs. topical application).

Other investigators reported that perineural injection of clonidine at or after the time of nerve injury produced antihypersensitivity effects that were slow in onset (days) and long in duration (weeks), likely through an immunomodulatory mechanism. Because systemic or intrathecal clonidine at the same doses had short-lasting or no effect on hypersensitivity, the local site of action of perineural clonidine was suggested. The previous and current studies suggest that topical clonidine has faster onset and shorter duration of action, compared with perineural clonidine, probably because topical clonidine acts mainly by suppressing neurotransmitter release and neuronal firing in neurons implicated in nociception, whereas perineural clonidine acts mainly through its immunomodulatory effects at the site of nerve injury, which may lead to suppression of the local neural inflammation.

In the current study, clonidine cream in a single dose effectively reduced thermal hyperalgesia and mechanical allodynia in the neuropathic pain model, whereas it had no such antihypersensitivity effects in the postoperative and inflammatory pain models. Furthermore, clonidine cream in repeated doses did not have antihypersensitivity effects either in the inflammatory pain model. In the postoperative pain model, however, clonidine cream in repeated doses reduced thermal hyperalgesia on the postoperative days, although it did not reduce mechanical allodynia throughout the 6-day observation period. Therefore, drug efficacy of clonidine cream could not reduce mechanical allodynia in the postoperative pain model, and it could not reduce hyperalgesia nor allodynia in the inflammatory pain model, despite spontaneous alleviation of these hypersensitive states over time.

Second, there is the possibility that diffusion of clonidine into deep tissue is limited in the postoperative and inflammatory pain models, due to the increased drug diffusion distance associated with inflammatory edema and due to the increased drug absorption into the blood associated with inflammatory hyperemia. It was also unlikely, however, that the increases in drug diffusion distance and drug absorption in these models could account for the diminished drug efficacy, because spontaneous alleviation of inflammation over time did not necessarily lead to increased drug efficacy in these pain models.

Third, one may suggest that licking the paw commonly observed after incision or during inflammation results in early removal of the cream, which causes diminished drug efficacy. This possibility seemed unlikely, however, because immediately after topical drug application, we prevented animals from licking the hind paws using a restrainer, and also because progressive decreases in licking behaviors over time did not necessarily lead to increased drug efficacy in these pain models.

Last, it has been shown that nerve injury causes significant alterations in α₂ adrenoceptors expressed on pain-transmitting neurons, which may result in increased sensitivity to α₂-adrenoceptor agonists in neuropathic pain. α₂-Adrenoceptor agonists, given systemically or spinally, are effective in hypersensitivity states in the opioid-insensitive rat SNL neuropathic pain model. Accumulating evidence suggests that nerve injury increases the potency and efficacy of α₂-adrenoceptor agonists in...
rodents\textsuperscript{15–17} and humans,\textsuperscript{1} possibly through various mechanisms, including up-regulation of descending inhibitory noradrenergic innervation to the lumbar dorsal horn,\textsuperscript{35} alterations in the proportion of subtypes of $\alpha_2$ adrenoceptors expressed on DRG and spinal dorsal horn neurons,\textsuperscript{36–37} alterations in the $\alpha_2$-adrenoceptor subtype activated by clonidine for analgesia,\textsuperscript{38} a shift of the site of $\alpha_2$-analgesic action to outside the central nervous system,\textsuperscript{10} concentrated peripheral $\alpha_2$ adrenoceptors at the site of peripheral nerve injury,\textsuperscript{9} and up-regulation of $\alpha_2C$ adrenoceptors in some injured medium and large DRG cells accompanied by increased proportion of clonidine-responsive cells.\textsuperscript{39} Taken together, it seemed most probable that clonidine cream in a single dose could reduce thermal hyperalgesia and mechanical allodynia only in rats with neuropathic pain, because potency and efficacy of $\alpha_2$-adrenoceptor agonists was increased after nerve injury.

Compared with neuropathic pain, clonidine cream was only partially effective in reducing hypersensitivity in the postoperative pain model. The mechanisms underlying the enhanced effect of $\alpha_2$-adrenoceptor agonists in neuropathic pain, such as modifications in $\alpha_2$-adrenoceptor subtypes, partly underlies incisional pain,\textsuperscript{40} and this may have contributed to the partial or limited anti-hypersensitivity effect of clonidine cream in rats with postoperative pain.

Our data indicate that clonidine cream has no and limited antihypersensitivity effects in acute inflammatory and postoperative pain, respectively. Fortunately, however, such types of acute pain are usually responsive to commonly used analgesics, including nonsteroidal anti-inflammatory drugs and opioids.\textsuperscript{2} In contrast, neuropathic pain is usually refractory to such analgesics.\textsuperscript{15–17} The potent antihypersensitivity activity of clonidine cream clearly demonstrated in neuropathic animals in the current study seems to be of great clinical relevance, because compared with systemic, intrathecal, or perineural administration, topical administration of clonidine cream is much easier to perform for both clinicians and patients, and perhaps associated with less adverse effects.\textsuperscript{3,4}

In conclusion, clonidine cream in a single dose was effective in reducing hypersensitivity and decreasing spinal Fos-like immunoreactivity in the rat neuropathic pain model, but not in the postoperative and inflammatory pain models. The antihypersensitivity effects of clonidine cream in neuropathic rats seemed to be mediated by peripherally located $\alpha_2$ adrenoceptors. Clonidine cream in multiple doses had only limited and no antihypersensitivity effects in the postoperative model and inflammatory pain model, respectively. These data add to the growing literature indicating that clonidine is effective in reducing hypersensitivity in neuropathic pain. The data also provide the rationale for clinical use of clonidine cream in patients with intractable neuropathic pain.

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