Intrathecal Clonidine Alleviates Allodynia in Neuropathic Rats

Interaction with Spinal Muscarinic and Nicotinic Receptors

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Background: Intrathecally administered clonidine increases release of spinal acetylcholine, which may be related to its analgesic action in neuropathic pain. The current study determined the role of spinal muscarinic and nicotinic receptors in the antiallodynic effect of intrathecally administered clonidine in spinal nerve-ligated rats.

Methods: Allodynia was produced in rats by ligation of the left 15-16 spinal nerves. Mechanical allodynia was determined by application of von Frey filaments to the left hindpaw. The effect of intrathecal injection of saline, two muscarinic receptor antagonists (atropine and scopolamine), and two nicotinic receptor antagonists (mecamylamine and hexamethonium) on the antiallodynic action produced by intrathecal administration of 20 μg clonidine was assessed in six groups of animals. Each group consisted of six to eight rats.

Results: Intrathecal injection of saline or muscarinic or nicotinic receptor antagonists did not alter the antiallodynic thresholds. The antiallodynic effect produced by intrathecally administered clonidine was attenuated in a dose-dependent manner by intrathecal treatment with muscarinic and nicotinic antagonists. Although nicotinic receptor antagonists only partially attenuated the effect of clonidine, blockade of spinal muscarinic receptors almost abolished the antiallodynic effect of clonidine.

Conclusions: These results demonstrate that the analgesic effect of intrathecally administered clonidine on neuropathic pain is mediated by spinal muscarinic and nicotinic receptors. Therefore, this study provides functional evidence that spinally released acetylcholine plays a role in the antiallodynic effect of intrathecally administered clonidine in neuropathic pain. (Key words: Acetylcholine; α2-adrenergic receptors; cholinergic receptors; neuropathic pain; nitric oxide; spinal cord.)

PERIPHERAL nerve injury caused by surgery or trauma is associated with spontaneous pain, hyperalgesia (i.e., increased pain intensity in response to noxious stimuli), and allodynia (i.e., normally innocuous stimuli become painful). These symptoms, common in patients with neuropathic pain, are often poorly relieved by conventional treatments such as nonsteroidal antiinflammatory drugs and opioids. The spinal cord is a major site of transmission of noxious sensory information by pre- and postsynaptic mechanisms. Clinical and experimental studies have demonstrated that intrathecal injection of clonidine, an α2-adrenergic receptor agonist, is an effective treatment of neuropathic pain conditions.

The mechanisms underlying the analgesic action of intrathecally administered clonidine are not fully known. It was proposed previously that the antiallodynic effect of intrathecally administered clonidine may be attributable to its inhibitory effect on sympathetic outflow. A recent study by Ossipov et al., however, found that the antiallodynic effect of clonidine is unrelated to sympathetic stimulation caused by clonidine in a rat model of neuropathic pain. We reported previously that intrathecally administered clonidine increases concentrations of acetylcholine in cerebrospinal fluid and microdialyses from spinal cord dorsal horn. Further, intrathecal administration of cholinomimetic agents produces analgesia, which is blocked by muscarinic receptor antagonists. Like many other tissues, the spinal cord...
contains muscarinic and nicotinic cholinergic receptors. Specific neuronal nicotinic receptor agonists also are known to produce analgesia in animal models of acute and chronic pain. Although data from previous neurochemical studies suggest that spinally released acetylcholine may be involved in the analgesic action of clonidine, there is a lack of behavioral evidence to support the functional importance of spinal muscarinic and nicotinic receptors in the analgesic action of intrathecally administered clonidine in neuropathic pain. It remains uncertain whether and to what extent spinal muscarinic/nicotinic receptors contribute to analgesia produced by intrathecally administered clonidine in neuropathic pain. Therefore, in the current study, we tested a hypothesis that spinal muscarinic and nicotinic receptors play a role in the antiallodynic effect of intrathecally administered clonidine in a rat model of neuropathic pain.

Materials and Methods

Male rats (Harlan Sprague-Dawley) weighing 150–180 g were used in this study. Ligation of L5 and L6 spinal nerves in rats was used in this study as an experimental model of neuropathic pain because it produces profound and sustained tactile allodynia, which resembles the condition observed in patients with neuropathic pain. During halothane-induced anesthesia, the left L5 and L6 spinal nerves were isolated and ligated tightly with 4-0 silk suture, according to the method described by Kim and Chung. The animals were allowed to recover for 5-7 days before intrathecal cannulation. Intrathecal catheters were inserted in rats during halothane-induced anesthesia as previously described by Yaksh and Rudy. Intrathecal catheters (PE-10 polyethylene tubing) were advanced 8 cm caudal through an incision in the epi-areal membrane and secured to the musculature at the incision site. Location of the catheter tip was confirmed in some (22 of 56) animals by postmortem dissection. Only animals with no evidence of neurologic deficits after catheter insertion were studied. All the pharmacologic experiments were conducted 3-4 weeks after spinal nerve ligation because tactile allodynia develops within 1 week after surgery and lasts for ≥6-8 weeks. The surgical preparations and experimental protocols were approved by the Animal Care and Use Committee at Wake Forest University School of Medicine.

The mechanical threshold was determined before and after spinal nerve ligation in all animals. To quantify mechanical sensitivity of the paw, rats were placed in individual plastic boxes on a mesh floor and allowed to acclimate for 30 min. A series of calibrated von Frey filaments (0.2-28.4 g; Stoelting Co., Wood Dale, IL) were applied perpendicularly to the plantar surface of the left paw with sufficient force to bend the filaments for 6 s. brisk withdrawal or paw flinching were considered positive responses. In the absence of a response, the filament of next greater force was applied. In the presence of a response, the filament of next lower force was applied. The tactile stimulus producing a 50% likelihood of withdrawal was determined using the “up-down” calculating method, as described in detail by Chaplan et al. Each trial was repeated two or three times at ~2-min intervals, and the mean value was used as the force to produce withdrawal responses.

After acclimation, baseline thresholds of withdrawal response to stimulation by von Frey filaments were determined. To determine the dose-response effect of muscarinic and nicotinic antagonists on the analgesic effect of intrathecally administered clonidine, animals were given 20 μg of clonidine intrathecally followed by intrathecal injection of saline (n = 6) or cumulative doses of atropine (5-45 μg, n = 7) or mecamylamine (5-100 μg, n = 7). The mechanical thresholds were determined every 15 min after each intrathecal injection. Our previous study showed that intrathecal injection of 20 μg of clonidine produces a 50% return to prenerve ligation withdrawal threshold in this animal model, and this effect lasts ~2 h. The dose ranges of muscarinic and nicotinic receptor antagonists were selected based on our preliminary data and previous studies related to their intrathecal use.

To ensure the specificity of the effect produced by blockade of spinal muscarinic and nicotinic receptors (atropine and mecamylamine), two structurally different muscarinic and nicotinic receptor antagonists, scopolamine and hexamethonium, also were studied. Animals were pretreated with intrathecal injection of saline (n = 6), 30 μg of atropine (n = 6), 30 μg of scopolamine (n = 8), 50 μg of mecamylamine (n = 7), or 50 μg of hexamethonium (n = 6), followed 15 min later by intrathecal injection of 20 μg of clonidine. The mechanical thresholds were determined before and 15, 30, 45, 60, and 120 min after treatment. It has been shown that the dose of atropine used in this study attenuates the antinociceptive effect of intrathecally administered carbamazepine. The doses of mecamylamine and hexamethonium selected for this study are known to inhibit similarly the pressor response caused by intrathecally administered nicotine.
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Fig. 1. Dose-dependent attenuation of the effect of intrathecal administered clonidine by treatment with atropine. Data are mean ± SD. *P < 0.05 compared with the threshold in the saline group after injection of clonidine.

in rats.25 In addition, we observed in the pilot study that the doses of the antagonists discussed here maximally inhibited the analgesic effect of clonidine.

Drugs for intrathecal injection were dissolved in normal saline and administered in a volume of 5 μl, followed by a 10 μl flush with normal saline.24,25 Clonidine, atropine, scopolamine, mecamylamine, and hexamethonium were obtained from Sigma Chemical Co. (St. Louis, MO).

Data are presented as mean ± SD. Paw withdrawal thresholds in response to mechanical stimulation before and after nerve ligation were compared using a paired Student’s t test. Effects of individual drugs on allodynia were determined by analysis of variance followed by the Tukey’s or Dunnett’s post hoc test, as appropriate. A probability value <0.05 was considered to be statistically significant.

Results

In all the animals examined, the tip of the intrathecal catheter resided in the lumbar intrathecal space. Paw withdrawal threshold before spinal nerve ligation was 28.1 ± 1.8 g. The mechanical threshold decreased significantly (2.1 ± 0.3 g, P < 0.05) within 7 days after nerve ligation and was maintained stable for ≥6 weeks in all animals studied. After spinal nerve ligation, rats developed typical foot deformities and changes in behaviors, such as licking the left hindpaw, avoiding weight bearing, or holding the paw in a protected position.

Effect of Muscarinic Antagonists on the Analgesic Effect of Clonidine

Intrathecal injection of 20 μg of clonidine increased the withdrawal threshold significantly (figs. 1 and 2). All animals receiving intrathecal injection of 20 μg clonidine exhibited sedation and diuresis as described previously.22 The antiallodynic effect of intrathecally administered clonidine reached maximum within 15 min and remained stable for ≥120 min (figs. 1 and 2). Repeat intrathecal injection of saline did not alter the allodynic state of nerve-injured rats (fig. 1). Intrathecal injection of 5–45 μg atropine inhibited the antiallodynic effect of intrathecal clonidine in a dose-dependent manner (fig. 1). Intrathecal injection of atropine and scopolamine did not change the baseline withdrawal thresholds (fig. 2). Similar to the effect observed with intrathecal administration of 45 μg atropine, pretreatment with 30 μg atropine or 30 μg scopolamine intrathecally almost completely blocked the effect of intrathecally administered clonidine (fig. 2).

Fig. 2. Effect of intrathecal pretreatment with saline, atropine (ATR), or scopolamine (SCP) on the time course of the antiallodynic effect of intrathecal injection of 20 μg clonidine. Data are mean ± SD. *P < 0.05 versus respective pretreatment control. **P < 0.05 compared with the threshold in the saline group after injection of clonidine.

Effect of Nicotinic Antagonists on the Analgesic Effect of Clonidine

Intrathecal administration of 5–100 μg of mecamylamine reduced the antiallodynic effect of intrathecally administered clonidine significantly in a dose-dependent manner (fig. 3). Unlike the effect of muscarinic antagonists, however, intrathecal injection of mecamylamine up to 100 μg only partially antagonized the analgesic effect of clonidine. Intrathecal pretreatment with 50 μg mecamylamine or 50 μg hexamethonium also partially attenuated the effect of clonidine, an inhibitory effect similar to that observed with 100 μg mecamylamine (fig. 4). Intrathecal injection of mecamylamine or hexamethonium neither changed the baseline withdrawal thresholds (fig. 4) nor produced any visible behavioral effects.

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Intrathecal administration of cholinergic receptor agonists or a cholinesterase inhibitor, neostigmine, produces analgesia in animals and in humans. Further, \( \alpha_2 \) and acetylcholine binding sites have been localized in the dorsal horn of the spinal cord, suggesting a possible interaction between \( \alpha_2 \) and acetylcholine receptor agonists in their analgesic effect. Direct involvement of muscarinic or nicotinic receptors in the antiallodynic effect of intrathecally administered clonidine, however, has not been demonstrated in neuropathic pain conditions. Using receptor autoradiography approaches, Hoglund and Baghdoyan have shown that the muscarinic receptor subtypes in the rat spinal cord are \( M_2 \), \( M_4 \), and \( M_4 \). But Naguib and Yaksh found that spinal \( M_1 \) or \( M_4 \) subtypes are responsible for the analgesic effect of intrathecally administered muscarinic receptor agonists. The difference between these two studies is likely attributable to the specificity of the agonists used because the currently available agonists for muscarinic receptor subtypes remain poorly selective, especially at high concentrations. In the current study, intrathecally administered atropine inhibited the effect of clonidine on allodynia in a dose-dependent manner. It is unlikely that the action of atropine is attributable to an unspecific effect because another muscarinic antagonist, scopolamine, antagonizes the analgesic effect of clonidine to the same extent observed with atropine. Thus, results from our study indicate that activation of spinal muscarinic receptors is important for manifestation of the analgesic action of intrathecally administered clonidine in neuropathic pain. These data are consistent with our recent observation that intrathecal administration of

**Discussion**

The role of spinal muscarinic and nicotinic receptors in the analgesic effect of intrathecally administered clonidine was investigated in the current study. We found that, in conscious behaving animals, intrathecal pretreatment with muscarinic or nicotinic receptor antagonists attenuated significantly the antiallodynic effect of intrathecally administered clonidine in a rat model of neuropathic pain. Although the nicotinic antagonists only partially inhibited the effect of clonidine, treatment with muscarinic antagonists essentially abolished the analgesic action of intrathecally administered clonidine. These data indicate that endogenously released acetylcholine evoked by intrathecally administered clonidine plays an important role in the analgesic action of clonidine in neuropathic pain. Therefore, our study provides new experimental evidence indicating that the antiallodynic effect produced by intrathecally administered clonidine in neuropathic pain depends on its interaction with muscarinic/nicotinic cholinergic receptors in the spinal cord.

Intrathecally administered clonidine has become an important alternative treatment for patients suffering from intractable pain conditions. It has been shown that intrathecally administered clonidine produces analgesia through spinal \( \alpha_2 \)-adrenergic receptors because intrathecal administration of a specific \( \alpha_2 \)-adrenergic receptor antagonist, yohimbine, abolished its action in nerve-ligated rats. Data from previous neurochemical studies suggest that acetylcholine is likely involved in the analgesic effect of clonidine. In this regard, it has been shown that intrathecally administered clonidine increases release of acetylcholine in the spinal cord.

**Fig. 3.** Dose-dependent attenuation of the effect of intrathecally administered clonidine by treatment with mecamylamine (mcm). Data are mean ± SD. *P < 0.05 compared with the threshold in the saline group after injection of clonidine.

**Fig. 4.** Effect of intrathecal pretreatment with saline, mecamylamine (MCM), or hexamethonium (HXM) on the time course of the antiallodynic effect of intrathecal injection of 20 \( \mu \)g clonidine. Data are mean ± SD. **P < 0.05 versus respective pretreatment control. ***P < 0.05 compared with the threshold in the saline group after injection of clonidine.
clonidine and neostigmine has a synergistic antiallodynic effect in neuropathic rats.22

There is no available evidence documenting a direct interaction between spinal α2-adrenergic and nicotinic cholinergic receptors in antinociception. In the rodent central nervous system, including the spinal cord, the predominant neuronal nicotinic receptor subtypes are α4β2, which differ from the α1β1, δγ(ε) and α5-containing nicotinic receptor subtypes found at the neuromuscular junction and sympathetic ganglia, respectively.17,18 Further, ABT-594, a preferential selective compound for neuronal α1β2 nicotinic receptors, produces analgesia in acute and chronic animal models of pain.18 Although it is possible that released acetylcholine evoked by intrathecially administered clonidine may act on muscarinic and nicotinic receptors, no previous studies have examined the role of spinal nicotinic receptors in the antinociceptive effect of clonidine. In the current study, we found that pretreatment with intrathecially administered nicotinic receptor antagonists also attenuated the antiallodynic effect of clonidine significantly in spinal nerve-ligated rats. We believe that attenuation of the effect of clonidine by nicotinic receptor antagonists is unlikely attributable to a nonselective effect because mecamylamine attenuated the antinociceptive effect of clonidine in a dose-dependent manner. Further, the two drugs, mecamylamine and hexamethonium, are structurally different and highly specific for the muscarinic receptors.23 Both agents demonstrated a similar inhibitory effect on the effect of clonidine in the current study. Therefore, data from the current study indicate that spinal nicotinic cholinergic receptors also contribute to the antinociceptive action of clonidine in neuropathic pain.

The current pharmacologic study demonstrates that, compared with the nicotinic receptors, the spinal muscarinic receptors play a greater role in the antinociceptive action of clonidine. Recent studies have provided substantial evidence indicating that spinal NO mediates the antinociceptive effect of systemic morphine and intrathecially administered clonidine in animal models of acute and chronic pain.24,25,26 A cascade of activation of α2-adrenergic receptors followed by release of acetylcholine and NO has been proposed to be a likely mechanism of the antinociceptive action of clonidine in the spinal cord.11 These data, together with previous observations,25,36 suggest that spinal muscarinic and nicotinic receptors, through interaction with NO, play a critical role in the antinociceptive effect of intrathecially administered clonidine in neuropathic pain (fig. 5).

In summary, we found that intrathecially administered analgesic mechanisms of intrathecially administered clonidine

clonidine alleviates allodynia produced by spinal nerve ligation in rats through interaction with spinal muscarinic and nicotinic receptors. Therefore, this study yields new information that intrathecially administered clonidine, through interaction with spinal muscarinic and, to a lesser extent, nicotinic receptors, produces its antiallodynic effect in neuropathic pain.

References


