Role of Spinal NO in Antiallodynic Effect of Intrathecal Clonidine in Neuropathic Rats

Hui-Lin Pan, M.D., Ph.D.,* Shao-Rui Chen, M.D.,† James C. Eisenach, M.D.‡

Background: The role of spinal nitric oxide (NO) in neuropathic pain remains uncertain. Although intrathecal clonidine causes NO release in the spinal cord, the functional role of spinal NO in clonidine-produced analgesia has not been examined. The objectives of this study were to assess the role of spinal NO in maintenance of allodynia and to determine the role of spinal NO in the antiallodynic effect of intrathecal clonidine.

Methods: Allodynia was produced in rats by tight ligation of the left 15-16 spinal nerves. Intrathecal catheters were inserted with tips in the lumbar intrathecal space. Mechanical allodynia was determined by application of von Frey filaments to the left hindpaw. In the first series of experiments, allodynia was assessed before and after intrathecal injection of saline, L-arginine, an NO donor (SNAP), two NO synthase inhibitors (TRIM and NMMA), or an NO scavenger (PTIO). In the second series of experiments, 20 μg of clonidine was injected intrathecally 15 min after intrathecal injection of saline, TRIM, NMMA, or PTIO.

Results: Allodynia was not affected significantly by intrathecal injection of L-arginine, SNAP, TRIM, NMMA, or PTIO. The antiallodynic effect produced by intrathecal injection of clonidine was attenuated significantly by pretreatment with TRIM, NMMA, or PTIO.

Conclusions: These results demonstrate that spinal NO neither contributes significantly to maintenance of allodynia nor produces detectable antiallodynic effect in this neuropathic pain model. Furthermore, this study provides functional evidence that spinal NO plays an important role in the antiallodynic effect of intrathecal clonidine in neuropathic pain. (Key words: Acetylcholine; α₂-adrenergic receptors; hyperalgesia; spinal cord.)

THE major symptoms of neuropathic pain are intense spontaneous pain, hyperalgesia (increased pain intensity in response to noxious stimuli), and allodynia (normally innocuous stimuli become painful). Because neuropathic pain is often unresponsive to conventional treatments, 1, 2 investigations into the basic mechanisms underlying neuropathic pain and development of new and alternative therapeutic interventions have generated considerable interest. The role of nitric oxide (NO) in the spinal cord in neuropathic pain remains uncertain. Although there are several lines of investigation suggesting that spinal NO is pronociceptive, 3–6 recent studies provide strong evidence that spinal NO is involved in antinociception produced by morphine. 7, 8 Because the role of spinal NO in the maintenance of neuropathic pain has not been fully studied, one objective of this study was to determine the effects of intrathecal administration of NO donors and NOS inhibitors on mechanical allodynia produced by spinal nerve ligation in rats.

Intrathecal injection of clonidine, an α₂-adrenergic receptor agonist, is effective for alleviating intractable pain conditions such as neuropathic pain. 9, 10 Intrathecal clonidine produces a significant antiallodynic effect in the L5-L6 spinal nerve-ligated rats. 11, 12 However, the mechanisms involved in its analgesic effect is not clear. Our previous neurochemical studies suggest that spinal NO may contribute to the analgesia action of intrathecally administered clonidine. We have shown that clonidine increases nitric oxide production in the rat spinal cord in vitro and in microdialysate samples from the dorsal horn of the sheep spinal cord. 13 Although clonidine is capable of increasing spinal cord NO release, no evidence is available to support the functional role of spinal NO in the analgesic actions of clonidine in conscious behaving animals. Thus, another objective of this study was to test a hypothesis that spinal NO contributes, at least in part, to the antiallodynic effect of intrathecal clonidine in a rat model of neuropathic pain.

Methods and Materials

Male rats (Harlan Sprague-Dawley) weighing 150–180 g were used in this study. Under halothane 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 given halothane anesthesia as previously described. Intrathecal catheters (PE-10 polyethylene tubing) were advanced 8 cm caudal through an incision in the cisternal membrane and secured to the musculature at the incision site. Location of the catheter tip was confirmed in some but not all animals by postmortem dissection. Only animals with no evidence of neurologic deficit after catheter insertion were studied. All the pharmacologic experiments were conducted between 3 and 4 weeks after spinal nerve ligation because tactile allodynia develops within 1 week after surgery and lasts for at least 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 von Frey filaments (filaments numbers 3.61 to 5.46, 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 as 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 2 or 3 times at approximately 2-min intervals, and the mean value was used as the force to produce withdrawal responses.

The first series of experiments was performed to determine the role of spinal NO in the maintenance of allodynia. After acclimation, baseline withdrawal thresholds to von Frey filament stimulation were determined. Next, the animals were given intrathecal injections, and then mechanical thresholds were determined 15, 30, 45, 60, and 120 min after treatment. L-Arginine (20–200 μg) or the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 20–200 μg) was injected intrathecally in a random order in six rats. The effects of intrathecal injection of the nonspecific NOS inhibitor, Nω-nitro-L-arginine (NMMA, 3–43 μg), the neuronal NOS specific inhibitor, 1-(2-trifluoromethylphenyl) imidazole (TRIM, 3–43 μg), or the NO scavenger, 2(4-carboxyphenoxy)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide potassium (PTIO, 3–45 μg) was randomly examined in seven separate neuropathic rats. Repeat intrathecal injections in the same animals were separated by at least three days. We have demonstrated recently that intrathecal NMMA, TRIM, or PTIO attenuates dose-dependently the analgesic effect of intravenous injection of morphine in rats.

In the second series of experiments, the role of spinal NO in the antiallodynic effect of intrathecal clonidine was examined in spinal nerve-ligated rats. Animals first received intrathecal injection of saline, 30 μg of NMMA, 30 μg of TRIM, or 50 μg of PTIO and followed in 15 min by intrathecal injection of 20 μg of clonidine. Our previous study has shown that this dose of clonidine produces a 50% return to presurgery withdrawal threshold in this animal model. The dose–response and duration of intrathecal NMMA, TRIM, and PTIO have been presented in our previous study, and doses of these compounds that maximally attenuated morphine-induced algesia in rats were used in the present study. To further determine the specificity of the inhibitory effect of NOS inhibitors on the antiallodynic action of intrathecal clonidine, two separate groups of rats were pretreated with intrathecal 30 μg of NMMA and followed by intrathecal injection of 20 μg of clonidine. Thirty minutes after intrathecal clonidine, 100 μg of L- or D-arginine was administered intrathecally. There were six or seven animals in each of six experimental groups.

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. D- and L-arginine, clonidine, and SNAP were obtained from Sigma Chemical Co. (St. Louis, MO). PTIO, NMMA, and TRIM were purchased from RBI (Natick, MA).

Data are presented as mean ± SD. Paw withdrawal thresholds 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 Tukey’s post hoc test. P < 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. After spinal nerve ligation, rats developed typical foot deformities and changes in behaviors, such as licking the left.
handpaw, avoiding weight bearing, or holding the paw in a protected position. Paw withdrawal threshold before spinal nerve ligation was 26.2 ± 1.8 g. The mechanical threshold decreased significantly (2.1 ± 0.3 g; \( P < 0.05 \)) within 7 days after nerve ligation and maintained stable for at least 6 weeks in all animals studied.

Neither intrathecal injection of L-arginine nor SNAP affected the paw withdrawal threshold in six animals (fig. 1). Furthermore, intrathecal injection of NMMA, TRIM, or PTIO did not alter significantly the mechanical withdrawal thresholds in seven separate rats (fig. 2). Animals were slightly excited (increased spontaneous activity) for 3–5 min after intrathecal injection of 100 \( \mu \)g of SNAP. There were no visible behavioral effects of intrathecal L-arginine, NMMA, TRIM, or PTIO.

Intrathecal injection of 20 \( \mu \)g of clonidine increased significantly the withdrawal threshold in six animals pre-

treated with intrathecal saline. The antiallodynic effect of intrathecal clonidine reached maximum within 15 min and maintained stable for at least 60 min (fig. 3). All animals receiving intrathecal injection of 20 \( \mu \)g of clonidine exhibited sedation and diuresis. Pretreatment with intrathecal NMMA, TRIM, or PTIO attenuated significantly the effect of intrathecal clonidine (fig. 3). Although specific measures were not used, the drugs did not appear to eliminate the side effects associated with clonidine administration. Additionally, in animals treated with intrathecal NMMA and clonidine, intrathecal administration of L- but not D-arginine reversed the inhibitory effect of NMMA on the antiallodynic effect of clonidine (fig. 4). In fact, the clonidine’s effect was enhanced significantly by intrathecal L-arginine compared with

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that seen in animals receiving intrathecal saline and clonidine.

Discussion

There are two major important observations from the present study. First, intrathecal injection of NO donors, NO synthase inhibitors, or an NO scavenger did not affect allodynia in the rat model of neuropathic pain. These data suggest that spinal NO is unlikely an important factor in the maintenance of allodynia in this animal model. Second, pretreatment with intrathecal NO inhibitors or an NO scavenger attenuated significantly the antiallodynic effect of intrathecal administration of a \( \alpha_2 \)-adrenergic receptor agonist, clonidine. Thus, our study provides the first behavioral evidence that spinal released NO plays an important role in mediating the analgesic effect of clonidine.

Several models of painful neuropathy have been developed in rats in recent years to study the mechanisms of development and maintenance of allodynia and to assess the effect of various treatments.\textsuperscript{14,20-21} Among these neuropathic pain models, ligation of L5 and L6 spinal nerves in rats has been widely used because it produces reliable and sustained tactile allodynia, which resembles the condition observed in patients with neuropathic pain.\textsuperscript{11,12,22} We examined initially the role of spinal NO in the maintenance of allodynia because the effects of intrathecal NO donors or NO inhibitors have not been investigated previously in this animal model of neuropathic pain. Overall, the role of spinal NO in nociception and antinociception is still controversial. Some evidence favors a pronociceptive action of spinal NO under many conditions. For example, in animal models of acute and chronic pain, there is an early upregulation of NOS in the spinal cord and an increased production of spinal NO after nerve constriction and tissue inflammation.\textsuperscript{4-6,22} Furthermore, intrathecal injection of NO inhibitors attenuates inflammation-induced hyperalgesia and allodynia in rats.\textsuperscript{3,6} and pretreatment with systemic NOS inhibitors delays the development of thermal hyperalgesia caused by sciatic nerve ligation.\textsuperscript{25} These data thus suggest a putative role of spinal NO in nociception. On the other hand, recent studies have provided strong evidence that spinal NO plays a role in antinociception. In this regard, systemic administration of the NO precursor, L-arginine, has been shown to produce analgesia in mice.\textsuperscript{24,26} Administration of NO donors inhibits neuronal activity in the superficial dorsal horn of rats.\textsuperscript{26,27} It also has been shown that neuronal NOS has two functionally different forms, representing two splice variants, in the spinal cord of mice.\textsuperscript{8} Selective reduction of one of the isoforms, NOS-2 (expressed mainly in the supraspinal site), by intrathecal administration of an antisense oligodeoxynucleotide inhibits the development of tolerance to morphine, whereas selective reduction of the other, NOS-1 (located predominantly in the spinal cord), blocks the analgesic action of morphine.\textsuperscript{8} Further, we have demonstrated that spinal NO largely mediates the analgesic action of systemic morphine in normal rats using noxious heat as a stimulus.\textsuperscript{1} However, the role of spinal NO in the maintenance of allodynia has not been investigated previously through intrathecal administration of NOS inhibitors or NO donors in neuropathic rats. In the present study, we found that intrathecal NO donors, NOS inhibitors, or an NO scavenger failed to alter the allodynic conditions in spinal nerve-ligated rats. These data are consistent with previous reports showing minimal or no antinociception from L-arginine or NO donors after intrathecal administration.\textsuperscript{7,26} It should be noted that our data do not exclude the possibility that spinal NO may play a role in early development of allodynia after spinal nerve ligation. It has been reported that pretreatment but not posttreatment with intrathecal NO inhibitors delays the development of thermal hyperalgesia induced by sciatic nerve constriction in rats,\textsuperscript{23} suggesting that spinal NO may contribute to the early development of hyperalgesia.

Previous studies have indicated that spinal \( \alpha_2 \)-adrenergic receptors are important for the analgesic action of morphine because intrathecal injection of \( \alpha_2 \)-adrenergic receptor antagonists decreases the effect of opioids.\textsuperscript{29} Although the analgesic effect of intrathecal \( \alpha_2 \)-adrenergic receptor agonists such as clonidine has been well demonstrated in animals and in humans,\textsuperscript{9,10,12} its underlying mechanisms have not been established. It was proposed previously that the antiallodynic effect of intrathecal clonidine may be a result of its inhibitory effect on sympathetic outflow.\textsuperscript{12} However, a recent study by Ossipov et al.\textsuperscript{11} has found that the antiallodynic effect of clonidine is not the result of sympatholysis caused by clonidine. We have shown that activation of \( \alpha_2 \)-adrenergic receptors in the spinal cord results in increased acetylcholine, which in turn stimulates NO synthesis, as evidenced by increased NO release from the rat spinal cord tissue \textit{in vitro} and microdialysates of the dorsal horn of the sheep spinal cord \textit{in vitro} after clonidine exposure.\textsuperscript{15,30} The cascade of spinal \( \alpha_2 \)-adrenergic receptors→acetylcholine→NO has been investigated in
our previous neurochemical studies, which demonstrate that specific $\alpha_2$-adrenergic receptor antagonists, muscarinic cholinergic receptor antagonists, and NOS inhibitors can block morphine and clonidine-induced NO release.\textsuperscript{13,30} But evidence for the involvement of spinal NO in analgesia produced by intrathecal clonidine has relied primarily on measurement of neurotransmitters in CSF or in spinal cord interstitial fluid by microdialysis. There is no behavioral evidence supporting the functional importance of spinal NO in the analgesic action of intrathecal clonidine. We found in the present study that inhibition of spinal NO blocked largely the antiallodynic effect of intrathecal clonidine. Thus, results from our study indicate that spinal NO is important for manifestation of the analgesic action of intrathecal clonidine. It is unlikely that attenuation of the effect of clonidine by NOS inhibitors is a result of a nonspecific action because intrathecal injection of two NOS inhibitors, one of which is considered to be selective for the neuronal NOS,\textsuperscript{18} as well as an NO scavenger, produced similar effects. Furthermore, we found that the inhibitory effect of NOS inhibitor, NMMA, on the antiallodynic effect of clonidine is reversed completely by L- but not D-arginine, which also supports the specificity of the effect of NOS inhibitors observed in our study. The current pharmacologic study in conscious rats reinforces our proposed mechanisms that activation of spinal presynaptic $\alpha_2$-adrenergic receptors represents a major mechanism of inhibitory control of nociception and that spinal NO is an important neurotransmitter mediating the analgesic effect of intrathecal clonidine. It is unclear why intrathecal injection of NO-releasing agents did not produce analgesia. Perhaps the interaction of spinal NO with co-released other chemicals, as a result of activation of $\alpha_2$-adrenergic receptors, is an important factor for clonidine’s analgesic effect. Further studies are warranted to examine the complex interplay between spinal NO and other neurotransmitters that produce analgesia related to activation of $\alpha_2$-adrenergic receptors.

In summary, we found that intrathecal administration of NOS inhibitors or NO donors does not affect allodynia caused by ligation of L5–L6 spinal nerves in rats. Furthermore, intrathecal pretreatment with NOS inhibitors or an NO scavenger decreases the antiallodynic effect of intrathecal clonidine in this rat model of neuropathic pain. These data, therefore, provide complementary functional evidence, indicating that intrathecal clonidine evoked-increases in spinal NO in animals and humans play an important role in the antiallodynic effect of clonidine in neuropathic pain.

References

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