Antiallodynic Effect of Intrathecal Neostigmine Is Mediated by Spinal Nitric Oxide in a Rat Model of Diabetic Neuropathic Pain

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Background: Intrathecal administration of acetylcholinesterase inhibitors produces antinociception in both animals and humans, but their effect on diabetic neuropathic pain has not been studied. In the current study, we determined the antiallodynic effect of intrathecal injection of an acetylcholinesterase inhibitor, neostigmine, in a rat model of diabetic neuropathic pain. In addition, since acetylcholine can increase release of nitric oxide in the spinal cord, we studied the role of spinal endogenous nitric oxide in the action of intrathecal neostigmine in diabetic neuropathic pain.

Methods: Rats were rendered diabetic with an intraperitoneal 50-mg/kg injection of streptozotocin. Intrathecal catheters were inserted, with tips in the lumbar intrathecal space. Mechanical allodynia was determined by application of von Frey filaments to the hind paw. We first determined the dose-dependent effect of intrathecal neostigmine on allodynia. The role of spinal nitric oxide in the action of intrathecal neostigmine was then examined through intrathecal treatments with a neuronal nitric oxide synthase inhibitor (TRIM), a nitric oxide scavenger (PTIO), L-arginine, or D-arginine.

Results: The diabetic rats developed a sustained tactile allodynia within 4 weeks after streptozotocin injection. Intrathecal injection of 0.1–0.5 μg neostigmine dose-dependently increased the withdrawal threshold in response to application of von Frey filaments. Intrathecal pretreatment with 30 μg TRIM or 30 μg PTIO abolished the antiallodynic effect of intrathecal neostigmine. Furthermore, the inhibitory effect of TRIM on the action of intrathecal neostigmine was reversed by intrathecal injection of 100 μg L-arginine but not D-arginine.

Conclusions: Intrathecal neostigmine produces a profound antinociceptive effect in a rat model of diabetic neuropathic pain. Spinal endogenous nitric oxide contributes to the analgesic action of intrathecal neostigmine in this rat model of diabetic neuropathic pain.

Diabetic neuropathy is one of the most common late complications of diabetes mellitus and is frequently painful, with the pain involving predominantly the distal extremities. Pain associated with diabetic neuropathy can occur either spontaneously or as a result of exposure to only mild painful stimuli (hyperalgesia) or to stimuli not normally perceived as painful (allodynia). Since diabetic neuropathic pain often is not adequately relieved by classical analgesics, it represents an important unmet medical need. Previous studies have demonstrated that activation of the spinal cholinergic system produces antinociception. First, intrathecal injection of muscarinic or nicotinic receptor agonists is effective to relieve acute and chronic pain in animals. Second, intrathecal injection of acetylcholinesterase inhibitors, such as neostigmine, also produces antinociception in both animals and humans. In addition, we have shown that the spinal cholinergic neurons and cholinergic receptors play an important role in the analgesic action of intrathecal α2 receptor agonists and intravenous morphine. In this regard, blockade of spinal muscarinic and nicotinic receptors or inhibition of the spinal high-affinity choline transporter attenuates the analgesic effect of intrathecal clonidine and systemic morphine in normal and neuropathic rats. The analgesic action of intrathecal acetylcholinesterase inhibitors has been shown in the rat model of neuropathic pain caused by spinal nerve ligation. However, the etiology and the mechanisms of neuropathic pain caused by diabetes differ from those induced by traumatic nerve injury. For example, it has been shown that allodynia induced by nerve ligation is predominantly mediated by myelinated afferents. On the other hand, diabetic neuropathic pain appears to be due to hypersensitivity of damaged unmyelinated C-fibers. Thus, in the current study, we determined the antiallodynic effect of intrathecal neostigmine in a rat model of diabetic neuropathic pain.

Recent studies have shown that generation of nitric oxide in the spinal cord is important for full manifestation of the analgesic action of intrathecal clonidine and systemic morphine. For example, treatment with nitric oxide synthase inhibitors or nitric oxide scavengers attenuates the analgesic effects of intrathecal clonidine and intravenous morphine. Acetylcholine is capable of increasing spinal cord nitric oxide release. It is possible that intrathecal neostigmine can produce analgesia on diabetic neuropathic pain by increasing acetylcholine accumulation in the spinal cord, which augments nitric oxide release in the spinal cord. However, there is no direct functional evidence supporting the role of spinal nitric oxide in the analgesic action of intrathecal cholinomimetic agents. Therefore, in the current study, we tested a hypothesis that spinal endogenous nitric oxide contributes, at least in part, to the antiallodynic effect of intrathecal neostigmine in conscious-behaving rats with diabetic neuropathic pain.
Materials and Methods

Male rats (Harlan Sprague-Dawley, Indianapolis, IN) initially weighing 225–250 g were used in this study. The surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine (Hershey, PA). Diabetes was induced by a single 50-mg/kg intraperitoneal injection of streptozotocin (STZ; Sigma Chemicals, St. Louis, MO) freshly dissolved in 0.9% sterile saline. Two weeks later, diabetes was confirmed in streptozotocin-injected rats by measuring plasma glucose concentrations in samples obtained from the tail vein. The glucose level was assayed enzymatically with diagnostic glucose reagents (Sigma, St. Louis, MO), and the colorimetric absorbance readings were done at 450 nm with use of a microplate spectrophotometer (SPECTRAMax Plus; Molecular Devices, Sunnyvale, CA). Only rats with blood glucose concentrations greater than 350 mg/dl and mechanical withdrawal thresholds less than 6 g (see below) were further used in this study. This experimental model of diabetic neuropathic pain has been described as a relevant model of chronic pain, with alterations of pain sensitivity and poor response to opioids administered systemically or intrathecally.

The diabetic rats were anesthetized with 2% halothane during surgical implantation of intrathecal catheters. The catheters were inserted through an incision in the cisternal membrane and advanced 8 cm caudally so that the tip of each catheter was positioned at the lumbar spinal level. The intrathecal catheters were externalized to the back of the neck and sutured to the musculature and skin at the incision site. After a 5- to 7-day recovery following cannulation, the rats underwent the behavior testing. All the final pharmacologic experiments were conducted between 4 and 7 weeks after streptozotocin injection. Previous studies and our pilot experiments have demonstrated that after streptozotocin injection, rats display a reproducible mechanical allodynia/hyperalgesia within 4 weeks, lasting for at least 7 weeks.

The mechanical threshold was determined before and 4 weeks after streptozotocin injection in all animals. The behavioral testing was conducted between 8:30 and 11:30 AM. To quantify mechanical sensitivity of the hind paw, rats were placed in individual plastic boxes on a mesh floor and allowed to acclimate for 30–45 min. A series of calibrated von Frey laments (1.0, 2.0, 4.0, 6.0, 8.0, 15, 26, and 56 g; Stoelting, Wood Dale, IL) were applied perpendicularly to the plantar surface of the left hind paw with sufficient force to bend the filaments for 6 s. Brisk withdrawal or paw flinching was considered a positive response. 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 by means of the “up-down” calculating method, as described in previous studies. Each trial was repeated 2–3 times at approximately 2-min intervals, and the mean value was used as the force to produce withdrawal responses. Motor function was evaluated by the placing-stepping reflex and the righting reflex. The former was evoked by drawing the dorsum of either hind paw across the edge of the table. The latter was assessed by placing the rat horizontally with its back on the table, which normally gives rise to an immediate coordinated twisting of the body to an upright position. Changes in motor function were scored as follows: 0, normal; 1, slight deficit; 2, moderate deficit; and 3, severe deficit.

We first determined the dose-response effect of intrathecal neostigmine on allodynia in rats with diabetes. After acclimation, baseline withdrawal thresholds to von Frey filament stimulation were determined. The animals were then given intrathecal injection of neostigmine, and the mechanical threshold to von Frey filament stimulation was determined at 15, 30, 45, 60, 120, and 180 min. The antiallodynic effect of intrathecal neostigmine (0.1, 0.2, and 0.5 μg) was tested in 6 diabetic rats. Subsequent intrathecal injections in the same animals were separated by at least 3 days. In the pilot experiments, we observed that intrathecal neostigmine produced no effect on allodynia at doses less than 0.1 μg, and intrathecal neostigmine caused evident side effects (such as diuresis and increased motor activity) when the doses were increased to 1 μg in this animal model.

Next we studied the role of spinal nitric oxide in the antiallodynic effect of intrathecal neostigmine in separate diabetic rats. Animals first were injected intrathecally with 30 μg 1-(2-trifluoromethyl)imidazole (TRIM, a selective neuronal nitric oxide synthase inhibitor; n = 8), 30 μg 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium (PTIO, a specific nitric oxide scavenger; n = 7), or saline (vehicle control; n = 6), followed in 15 min by intrathecal injection of 0.2 μg neostigmine. This dose of neostigmine produced an approximately 85% return to the withdrawal threshold in rats before streptozotocin injection (see Results). The doses of intrathecal TRIM and PTIO were effective in attenuating the algic actions of intravenous morphine and intrathecal clonidine in our previous studies. The withdrawal threshold in response to application of von Frey filaments was then tested every 15–30 min for 180 min. To examine whether spinal nitric oxide plays a role in the maintenance of allodynia associated with diabetic neuropathy in rats, 30 μg TRIM or 30 μg PTIO was injected intrathecally in five additional diabetic rats.

To further ensure the specificity of the nitric oxide synthase inhibitor used, we determined whether the nitric oxide precursor, l-arginine, could reverse the inhibitory effect of TRIM on the antiallodynic action of intrathecal neostigmine. Additional diabetic rats were
intrathecally injected with 30 μg TRIM plus 0.2 μg neostigmine. Thirty minutes after intrathecal injection of neostigmine and TRIM, 100 μg L-arginine (n = 7) or 100 μg D-arginine (n = 6) was administered intrathecally. The withdrawal threshold in response to application of von Frey filaments was then tested every 15–30 min for 180 min. In addition, in five separate diabetic rats, the effect of intrathecal injection of 100 μg L-arginine alone on allodynia was examined.

Drugs for intrathecal injections 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 and neostigmine were obtained from Sigma Chemical, and PTIO and TRIM were purchased from Research Biochemical International. All the data collected (withdrawal thresholds) with the up-down method were normally distributed, as determined by the Komogorov–Smirnov test. Thus, the data are presented as mean ± SEM, and parametric tests were chosen for statistical analysis. Paw withdrawal thresholds in response to mechanical stimulation before and after streptozotocin injection were compared with use of a paired Student t test. Effects of individual drugs on allodynia were determined by repeated measures analysis of variance, followed by Tukey post hoc test. P < 0.05 was considered to be statistically significant.

Results

Most rats (87%) developed hyperglycemia within 2 weeks after streptozotocin injection. The diabetic rats displayed polyuria, a reduced growth rate, and a marked increase in food and water intake. The mechanical threshold before streptozotocin treatment was 28.6 ± 0.9 g in all rats used in this study. The mechanical threshold decreased significantly (4.2 ± 0.4 g; P < 0.05) 4 weeks after streptozotocin injection and lasted for at least 7 weeks. Four of 38 diabetic rats failed to develop stable allodynia (withdrawal threshold, greater than 6 g) 5 weeks after streptozotocin injection and were not used in this study.

Intrathecal injection of 0.1–0.5 μg of neostigmine increased significantly the mechanical withdrawal threshold in six animals in a dose-dependent fashion (fig. 1). The effect of intrathecal neostigmine on allodynia reached maximum within 15 min and gradually returned to baseline within 2–4 h. Intrathecal administration of neostigmine, at a dose of up to 0.5 μg, was not associated with motor function changes, as assessed by testing the animals’ ability to stand and ambulate in a normal posture and to place and step with the hind paw. All rats received a score of 0 after intrathecal injection of 0.1–0.5 μg neostigmine. We did not observe other visible behavioral changes, such as sedation or agitation, in rats receiving the above doses of neostigmine.

In rats treated with intrathecal saline, intrathecal injection of 0.2 μg neostigmine increased significantly the withdrawal threshold, and the effect lasted for approximately 2 h (fig. 2). On the other hand, intrathecal pretreatment with 30 μg TRIM or 30 μg PTIO eliminated completely the antiallodynic effect of intrathecal injection of 0.2 μg neostigmine (fig. 2). Neither 30 μg PTIO nor 30 μg TRIM injected intrathecally affected significantly the baseline withdrawal threshold. The paw withdrawal threshold was 4.6 ± 0.9 g before intrathecal injection. The withdrawal threshold was 4.4 ± 0.8 and 4.7 ± 0.9 g, respectively, 30 min after intrathecal administration of PTIO and TRIM. There were no visible behavioral effects following intrathecal administration of PTIO or TRIM.

In animals treated intrathecally with 30 μg TRIM plus 0.2 μg neostigmine, subsequent intrathecal administration of 100 μg L-arginine reversed the inhibitory effect of TRIM on the antiallodynic effect of neostigmine (fig. 3). By contrast, 100 μg D-arginine did not alter significantly the inhibitory effect of TRIM on the effect of neostig-
Discussion

In the current study, we determined the role of spinal nitric oxide in the effect of intrathecal neostigmine on allodynia in a rat model of diabetic neuropathic pain. We found that intrathecal neostigmine produced an antiallodynic effect in a dose-dependent manner in this animal model of diabetic neuropathic pain. In addition, we demonstrated that pretreatment with a selective neuronal nitric oxide synthase inhibitor, TRIM, or a specific nitric oxide scavenger, PTIO, abolished the antialgesic action of intrathecal neostigmine. Furthermore, the inhibitory effect of TRIM on the effect of intrathecal neostigmine was effectively reversed by l-arginine but not by d-arginine. Thus, these data provide strong evidence that intrathecal neostigmine produces an antialgesic effect on diabetic neuropathic pain through generation of nitric oxide in the spinal cord.

The spinal cholinergic system is one of the important targets for development of novel analgesics. The importance of such an analgesic system is exemplified by the following findings: (1) cholinergic neurons and muscarinic and nicotinic receptors are present in the spinal cord,26–28 (2) intrathecal administration of cholinergic agonists and acetylcholinesterase inhibitors produces analgesic effects in both animals and humans,7,8,12 and (3) the spinal endogenous cholinergic system is important for the analgesic action of intravenous morphine and intrathecal clonidine.9,10 Neostigmine is a selective inhibitor of acetylcholinesterase, which decreases the degradation of acetylcholine and increases local acetylcholine accumulation. The spinal acetylcholine appears to be produced within the spinal cord because neurons containing choline acetyltransferase are located in the spinal cord. Hwang et al.11 found that the antiallodynic effect of intrathecal acetylcholinesterase inhibitors is mediated by both muscarinic and nicotinic receptors in rats subjected to L5 and L6 spinal nerve ligation. Although systemic administration of a cholinergic channel modulator is effective on mechanical hyperalgesia in the rat model of diabetic neuropathic pain,5 the effect of intrathecal muscarinic and nicotinic agonists or acetylcholinesterase inhibitors on diabetic neuropathic pain has not been studied previously. We demonstrated that, in the rat model of diabetic neuropathic pain, intrathecal neostigmine produced a profound antiallodynic effect with minimal detectable side effects in the doses used in the current study. We observed that intrathecal injection of higher doses of neostigmine increased the withdrawal threshold greater than the baseline. Since intrathecal neostigmine is capable of producing analgesia in normal animals,6,8 this observation suggests that higher doses of neostigmine not only reversed allodynia but also produced an analgesic effect in this model. It should be noted that in this study, the doses of intrathecal neostigmine to produce antiallodynia are much less than those used in a rat neuropathic pain model caused by spinal nerve ligation.11,12 We are uncertain about the causes of the increased analgesic potency of intrathecal neostigmine in the rat model of diabetic neuropathic pain, although it may be due to the altered acetylcholine metabolism or cholinergic receptors in the spinal cord in diabetes. Future experimental and clinical studies are warranted to further substantiate this finding.

Our study provides new evidence that spinal nitric oxide mediates the analgesic action of intrathecal neostigmine in diabetic neuropathic pain. Recent studies have shown that spinal endogenous nitric oxide is an important mediator for the analgesic action of intravenous morphine and intrathecal clonidine.16,17 The functional link between acetylcholine and nitric oxide in the spinal cord has been implicated in previous studies. In this regard, it has been documented that cholinergic neurons are co-localized with neurons containing NADPH diaphorase in the spinal cord dorsal horn.28,29 Perfusion with acetylcholine increases nitric oxide production in the rat spinal cord.18 Acetylcholine also mediates nitric oxide release stimulated by morphine and clonidine in the spinal cord.30,31 Although acetylcholine is capable of increasing spinal cord nitric oxide release, no functional evidence has been presented to support the role of spinal nitric oxide in the analgesic actions of cholinomimetic agents in conscious-behaving animals. In the current study, we found that pretreatment with a neuronal nitric oxide synthase inhibitor, TRIM,24 or a specific nitric oxide scavenger, PTIO,25 eliminated the
antiallodynic effect of neostigmine in a rat model of diabetic neuropathic pain. Furthermore, we demonstrated that intrathecal L-arginine but not D-arginine reversed the inhibitory effect of TRIM on the antiallodynic action of intrathecal neostigmine. Therefore, the current study provides substantial evidence that spinal endogenous nitric oxide is essential for the antiallodynic effect of intrathecal neostigmine in the rat model of diabetic neuropathic pain.

Similar to what has been reported previously,\textsuperscript{16,52} we observed that intrathecal TRIM and PTIO alone did not affect the allodynic state in this animal model of neuropathic pain. This observation suggests that spinal nitric oxide is not responsible for the maintenance of allodynia associated with diabetic neuropathy.\textsuperscript{52} Furthermore, since intrathecal L-arginine had no effect on the allodynic condition, the exogenously introduced nitric oxide seems ineffective in producing evident analgesia, consistent with what has been reported previously.\textsuperscript{16} It should be acknowledged that there is evidence suggesting that spinal nitric oxide may play a role in the generation of pain. In this regard, intrathecal administration of nitric oxide synthase inhibitors attenuates hyperalgesia and allodynia caused by inflammation in rats.\textsuperscript{55} In addition, pretreatment with nitric oxide synthase inhibitors delays the development of thermal hyperalgesia caused by sciatic nerve ligation.\textsuperscript{54} At present it is difficult to explain the distinct roles of spinal nitric oxide in nociception caused by inflammation and nerve injury and antinoceptive actions produced by intrathecal neostigmine. Different nitric oxide species and their interactions with other substances in the spinal cord may be involved in the cholinergic analgesia observed in this study. For instance, we recently have shown that spinal nitric oxide can interact with the thiol-containing compounds to form S-nitrosothiols to produce an antiallodynic effect in a rat model of neuropathic pain.\textsuperscript{55} Future studies are required to further explore the interaction between nitric oxide and other chemicals in the spinal cord and their effect on spinal sensory neurons. Our data do not exclude the possibility that spinal nitric oxide may play a role in early development of allodynia and hyperalgesia induced by nerve injury. It has been shown that pretreatment but not posttreatment with intrathecal nitric oxide inhibitors delays the development of thermal hyperalgesia induced by sciatic nerve constriction in rats,\textsuperscript{54} suggesting that spinal nitric oxide may contribute to the early development of hyperalgesia.

Collectively, the current study indicates that enhancement of the spinal cholinergic system by intrathecal neostigmine effectively relieves allodynia associated with diabetic neuropathic pain in rats. Furthermore, our results provide additional evidence that spinal endogenous nitric oxide is an important mediator for the analgesic action of intrathecal cholinergic agonists or acetylcholinesterase inhibitors in the rat model of diabetic neuropathic pain. This study suggests that intrathecal neostigmine and nitric oxide donors may have some unique value in treatment of diabetes-associated neuropathic pain in humans.

References

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