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Effects of Intrathecal Neostigmine, Bupivacaine, and Their Combination on Sympathetic Nerve Activity in Rats

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Background: **Intrathecal injection of local anesthetic agents is associated frequently with hypotension. Conversely, intrathecal administration of neostigmine increases blood pressure by enhancing the accumulation of acetylcholine in the spinal cord. The current study examined directly the interaction of intrathecal injection of bupivacaine and neostigmine on splanchnic sympathetic efferent nerve activity.**

Methods: **Experiments were performed in rats with intrathecal catheters implanted for the long-term. Rats were anesthetized with ketamine (40 mg/kg, intramuscularly) and α-chloralose (60 mg/kg, intraperitoneally). The skin incision sites were infiltrated with 1% lidocaine. Sympathetic efferent activity was recorded from the left greater splanchnic nerve. Sympathetic nerve activity was measured continuously before and after intrathecal injection of saline, 430 nmol (140 μg) of bupivacaine, 25 nmol (7.6 μg) of neostigmine, and a combination of bupivacaine and neostigmine all in volumes of 5 μl. Each group consisted of six animals.**

Results: **Compared with baseline nerve activity, intrathecal injection of neostigmine increased splanchnic sympathetic nerve activity significantly by (mean ± SEM) 112 ± 29% after an onset latency of 6.8 ± 0.9 min. In contrast, bupivacaine decreased splanchnic nerve activity significantly (−65 ± 13%) after a latency of 3.3 ± 0.5 min after intrathecal administration. Similar to the effect of saline, intrathecal coadministration of bupivacaine and neostigmine did not alter the splanchnic sympathetic nerve activity significantly.**

Conclusions: **The current study provides electrophysiologic evidence that intrathecal injection of neostigmine increases whereas bupivacaine decreases sympathetic nerve activity. Further, addition of neostigmine effectively counteracts the inhibitory effect of spinal bupivacaine on the sympathetic nerve activity. (Key words: Acetylcholine; local anesthetics; spinal cord; splanchnic nerve; sympathetic nervous system.)**

HYPOTENSION associated with spinal and epidural administration of local anesthetic agents remains a common clinical problem.[1–3] This hypotension is thought to be due to reduced sympathetic activity, although it has not been established whether this is from axonal block of sympathetic fibers or inhibition of spinal preganglionic neurons by local anesthetic agents.[1,4] One approach to minimize hypotension is to combine the local anesthetic with an agent that has excitatory effect on sympathetic preganglionic neurons.[4,5] Acetylcholine is an excitatory neurotransmitter for preganglionic sympathetic neurons, and it is known that intrathecal injection of cholinergic receptor agonists or cholinesterase inhibitors increases blood pressure presumably through augmentation of the sympathetic output.[6–8] Further, the spinal cholinergic system has been increasingly recognized for its role in modulation of nociception.[5,9] In this regard, neostigmine, an inhibitor of cholinesterase, has been introduced into clinical protocols for spinal analgesia.[10] One important aspect of neostigmine is its excitatory effect on the cardiovascular system by enhancement of local accumulation of acetylcholine in the spinal cord. It has been demonstrated that spinal administration of neostigmine lessened the hypotensive effect induced by intrathecal bupivacaine in conscious animals.[4] The interaction of these two drugs on the spinal pre-ganglionic sympathetic efferent nerve activity has not been determined directly, however.

The rationale of this study was that spinal administration of bupivacaine and neostigmine have opposite effects on sympathetic efferent nerve activity, and therefore intrathecal coadministration of these two drugs may result in minimal changes in sympathetic output. The splanchnic vasculature has been demonstrated to play a prominent role in the origin of hypotension from spinal anesthesia.[11] In the current study, therefore, we

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directly recorded splanchnic sympathetic efferent activity to test a hypothesis that addition of neostigmine attenuates the inhibitory effect of spinal administration of bupivacaine on sympathetic nerve activity.

Materials and Methods

Surgical Preparation

Experiments were performed on male Sprague-Dawley rats (weights, 300–340 g). The rats were housed in an environmentally controlled room on a 12-h light–12-h dark cycle. Surgical and experimental protocols used in this study were approved by the Animal Care and Use Committee at Wake Forest University School of Medicine. Rats were anesthetized with a combination of ketamine (40 mg/kg, intramuscularly) and xylazine (5 mg/kg, intramuscularly) and then oriented in a stereotaxic frame with the head flexed at a 45° angle. An intrathecal catheter (PE-10 tubing) was inserted through a small opening in the cisterna magnum and advanced 8.5 cm caudally so that the catheter tip was located in the lumbar region in the intrathecal space.[12] We confirmed the location of intrathecal catheters by dorsal laminectomy in pilot experiments. We routinely flushed the catheter with 100–200 μl of saline after immediate implantation to exclude the possibility that the tip of the catheter was inserted into the spinal tissue. The rostral end of the catheter was sealed, and the intrathecal catheter was then externalized over the back of the neck. After surgery, rats were housed individually with free access to food and water and allowed to recover for 3–5 days before nerve recording experiments. Rats showing postoperative motor or sensory deficits were killed immediately with an overdose of pentobarbital (200 mg/kg, intraperitoneally).

For nerve recording studies, anesthesia was induced again with ketamine (40 mg/kg, intramuscularly) and then maintained with α-chloralose (60 mg/kg, intraperitoneally). α-Chloralose was selected for this study because it has minimal effects on cardiovascular and respiratory systems and has a long-lasting anesthetic effect.[13] In pilot experiments, we were able to use this dose of α-chloralose to achieve stable and prolonged anesthesia during nerve recording periods. The femoral artery was cannulated with PE-50 tubing. Arterial blood pressure was measured with a pressure transducer and recorded on a Grass recorder (Grass Instruments, Quincy, MA). Body temperature was maintained in the range of 36–38°C with a circulating water heating pad and heat lamps. A flank incision was made to expose the left greater splanchnic nerve. The skin incision sites were infiltrated with a small amount (0.2–0.3 ml of 1% lidocaine. The purpose of this procedure was to minimize the reflex effect on sympathetic efferent activity caused by activation of cutaneous nociceptors after surgery. The greater splanchnic nerve was used because this nerve contains pre- and postganglionic sympathetic efferents innervating the splanchnic resistance and capacitance vessels, and tonic control of these vessels by the sympathetic nervous system likely plays a predominant role in maintaining normal baseline blood pressure.[11,14–16] The nerve was isolated from the surrounding tissue and then placed on a platform and covered with warm mineral oil. Small nerve filaments were dissected gently from the nerve under an operating microscope (M900; D. F. Vasconcellos S. A., São Paulo, Brazil). The central cut end of the nerve was placed across a bipolar recording electrode to prevent recording of visceral sensory nerve activity, as the greater splanchnic nerve consists of sympathetic afferent and efferent fibers.[13,17] As we described previously,[13,16] the recording electrode was connected to a high impedance probe (Grass Instruments). The nerve action potential was then amplified 10,000–20,000× (model P511 preamplifier; Grass Instruments), pass band filtered between 0.3 and 1.0 kHz, processed through an audioamplifier (AM8 Audio Monitor; Grass Instruments), and displayed on a storage oscilloscope (model 450; Gould, Cleveland, OH). The amplified nerve signals (usually containing a few active fibers) were digitized through an analog-to-digital data interface card (DT 2821; Data Translation, Inc., Marlboro, MA) and fed into a PC-based data acquisition and analysis system (DataWave Technology, Inc., Longmont, CO) for subsequent off-line quantitative analysis. The software used peak and trough acceptance criteria to differentiate and count the impulse frequency of efferent nerves. The nerve discharge frequency was counted using the DataWave software, and the histogram was subsequently constructed with graphic software (SigmaPlot, version 3.0; Jandel Scientific Software, San Rafael, CA). Accurate counting of the nerve discharge frequency was verified by comparing the constructed histogram with the recorded original neurogram.

Experimental Protocols

Once the nerve action potential was identified and satisfactorily separated from the background noise, the nerve discharge frequency was monitored for at least

30–45 min to ensure the stability of nerve activity before experimental interventions. Then, the splanchnic efferent nerve activity was continuously recorded for a 5-min control period and then for 20 min after intrathecal injection of drugs. Normal saline, 25 nmol (7.6 μg) of neostigmine, 430 nmol (140 μg) of bupivacaine, or the combination of neostigmine and bupivacaine all in volumes of 5 μl were injected intrathecally, and the intrathecal catheter was then flushed with 10 μl saline (the volume of intrathecal catheters was ≈10 μl). Neostigmine and bupivacaine (Sigma Chemical Co., St. Louis, MO) were dissolved in sterile saline. The *p*H levels of bupivacaine and neostigmine solutions were similar to that of saline (≈5.8). A previous study with this dose of bupivacaine in conscious rats demonstrated a spinal block extending to the forelimbs without affecting respiration.[4] In preliminary experiments, we also observed that the blood gas including P_{CO2} was not altered by intrathecal neostigmine or bupivacaine. The time period of nerve recording was selected based on our preliminary experiments and a previous report, which demonstrated that the effect of intrathecal administration of neostigmine or bupivacaine on blood pressure lasts <20 min in conscious rats.[4] Each experimental group consisted of six rats, and the animals were assigned randomly to each group. At the end of experiments, the animals were killed with an overdose of pentobarbital (200 mg/kg, intraperitoneally). In addition, to confirm whether the recorded sympathetic nerve activity originated from the spinal cord, 2% lidocaine (5 μl) was injected intrathecally in three of six animals in the saline control group.

Nerve discharge frequency was averaged every 60 s during the 5-min control period and during the 20 min after intrathecal injections. Latency of changes in efferent activity after administration of drugs was measured from the time of intrathecal injection to the time when sustained discharge frequency increased or decreased by 30%. Because the number of nerve fibers recorded vary among animals, the effects of intrathecal interventions on sympathetic nerve activity were expressed as the percent change in sympathetic nerve activity, where the mean value of sympathetic nerve activity during the control period was used as a normalizing factor.[11] Repeated-measures analysis of variance was used initially to compare raw data of discharge nerve activity. The Dunnett's *post hoc* test then was used to localize significant variances detected by the analysis of variance test before the data were normalized. All statistical analyses were performed using SigmaStat version 2.0 (Jandel Scientific Software). Differences were considered to be statistically significant if $P < 0.05$.

Results

Figure 1 is a representative histogram showing the effect of neostigmine on the splanchnic efferent nerve activity in one rat. After intrathecal injection of 25 nmol neostigmine, the splanchnic nerve activity gradually increased after a latency of ≈7 min. The discharge activity of the splanchnic nerve reached maximum at 11 min, then declined but remained higher than the control baseline activity in this rat.

The baseline discharge frequencies were 5.4 ± 0.7, 4.3 ± 0.9, 7.0 ± 1.2, and 10.2 ± 1.0 impulses per second per animal in saline, neostigmine, bupivacaine, and the neostigmine/bupivacaine groups, respectively. Figure 2 illustrates the time course of changes in normalized splanchnic nerve activity during the 20 min after intrathecal injections in four groups of rats. Intrathecal injection of neostigmine potentiated significantly the splanchnic nerve activity. Compared with baseline nerve activity, the maximal change of discharge activity of the splanchnic nerve was increased by 112 ± 29% ($P < 0.05$). The onset latency of the effect of neostigmine was 6.8 ± 0.3 min. In contrast, intrathecal injection of bupivacaine attenuated significantly the splanchnic sympathetic nerve activity (65 ± 13% reduction, $P < 0.05$) after a latency of 3.3 ± 0.2 min.

Intrathecal injection of saline did not alter splanchnic nerve activity significantly compared with the baseline nerve activity in six separate rats (fig. 2). In three rats, subsequent intrathecal injection of 2% lidocaine completely eliminated the splanchnic nerve activity within 5 min, confirming that the recorded nerve activity originated from the spinal cord.

The effect of intrathecal injection of a combination of 25 nmol neostigmine and 430 nmol bupivacaine on splanchnic nerve activity is shown in figure 2. After intrathecal injection of neostigmine/bupivacaine, the nerve discharge activity did not differ significantly from the baseline control ($P > 0.05$).

Discussion

The current study examined directly the effect of intrathecal injection of neostigmine and bupivacaine on sympathetic outflow by recording splanchnic nerve activity in anesthetized animals. This study provides elec-

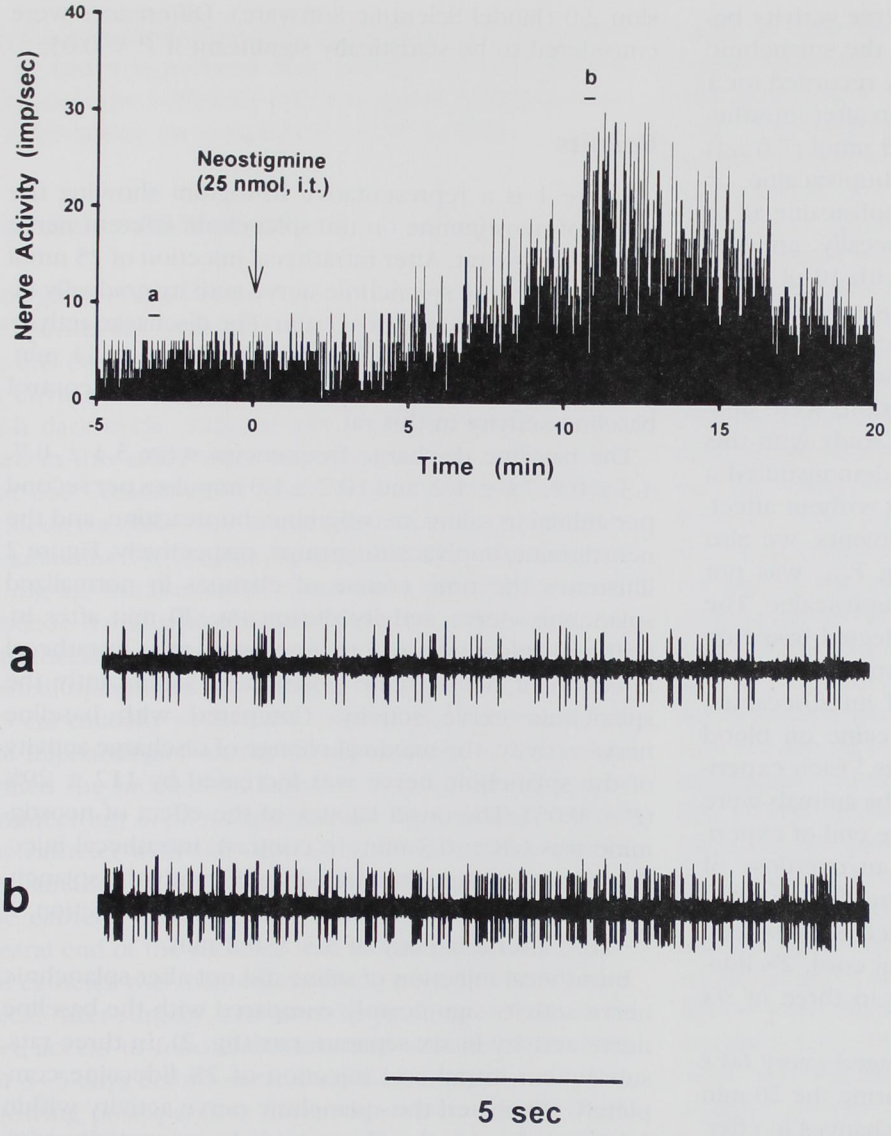

Fig. 1. Histogram of splanchnic nerve activity during a 5-min control period and 20 min after intrathecal injection of 25 nmol of neostigmine. *(A and B)* Representative original tracings of the efferent neurogram at times indicated by the bars above the histograms.

trophysiologic evidence that intrathecal injection of neostigmine increases, whereas bupivacaine decreases sympathetic nerve activity. Further, addition of neostigmine counteracts the inhibitory effect of spinal bupivacaine on the sympathetic nerve activity.

Hypotension is the most important consequence of extensive neural blockade during spinal anesthesia using local anesthetics.[1-4] This hemodynamic change generally has been considered to be due to the inhibitory effect of local anesthetic agents on the sympathetic outflow.[4,11] Local anesthetic agents have been known to inhibit the generation and the conduction of the nerve action potential through their action on sodium channels.[18] Direct measurement of sympathetic nerve activity provides a more sensitive approach to evaluate the effect of drugs injected spinally. We found that intrathecal injection of bupivacaine decreased the discharge activity of the splanchnic nerve significantly. Our finding is consistent with a previous study, which demonstrated that epidural lidocaine inhibits the sympathetic nerve activity in anesthetized rabbits.[11] The splanchnic vessels are richly innervated by sympathetic nerves, which tonically control both the resistance and capacitance vessels.[19,20] In addition, tonic sympathetic neural control of the splanchnic circulation plays a key role in maintaining circulatory homeostasis during physiologic

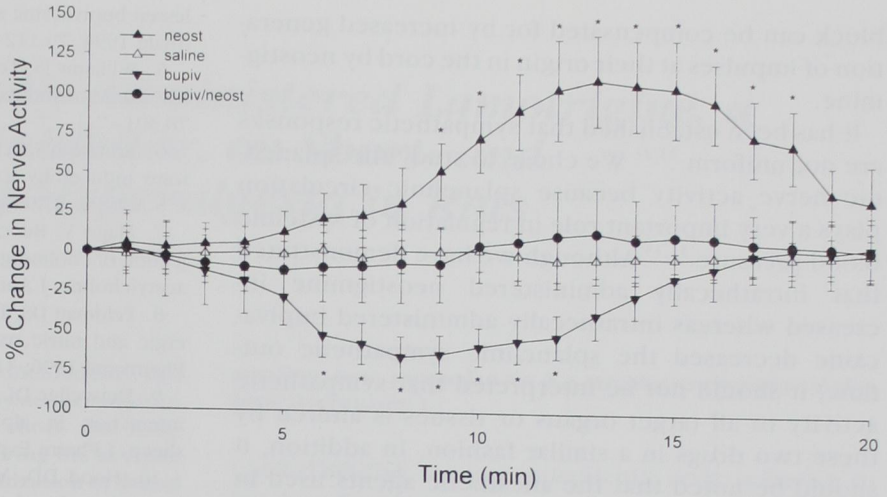

Fig. 2. Changes in splanchnic sympathetic nerve activity during the 20 min after intrathecal injection of saline, 25 nmol of neostigmine, 430 nmol of bupivacaine, or a combination of neostigmine/bupivacaine. Values are means ± SEM. *$P < 0.05$ compared with respective baseline values (time = 0).

conditions. Spinal or epidural injection of local anesthetics therefore attenuates tonic sympathetic discharge, and this sympatholytic effect induces peripheral dilatation of splanchnic resistance and capacitance vessels, consequently decreasing blood pressure.[11]

In contrast to the action of local anesthetic agents, it has been shown that intrathecal injection of neostigmine increases blood pressure.[4,7] The current study provides direct evidence that intrathecal injection of neostigmine is capable of initiating a significant increase in splanchnic nerve activity, which is responsible for increased blood pressure induced by intrathecal neostigmine. The mechanisms of the effect of intrathecal neostigmine on the splanchnic sympathetic nerve have not been completely established. Based on previous studies, neostigmine increases synaptic concentration of acetylcholine in the spinal cord, which may excite preganglionic sympathetic neurons through an action on muscarinic M_2 receptors located in the intermediolateral cell column of the spinal cord.[7,8,21,22] Nitric oxide also has been proposed recently to be an important mediator of the action of acetylcholine, because inhibition of the production of nitric oxide with L-NAME prevents the hemodynamic effect of intrathecal injection of cholinergic receptor agonists.[8] We observed that the onset latency for the effect of neostigmine is longer than that of bupivacaine. This disparity could be explained by the lesser lipophilicity of neostigmine, perhaps resulting in longer time to penetration of the spinal cord tissue for neostigmine than for bupivacaine. Further, the greater splanchnic nerve mainly originates from the preganglionic neurons in the thoracic spinal region.[17] Although the tip of the catheter was located in the lumber segment of the spinal cord, the injectate can diffuse to the thoracic level to access the preganglionic neurons.[22] In the current study, the sympathetic activity was measured in both pre- and postganglionic nerves. Further, it has been demonstrated that systemic administration of 25 nmol neostigmine, unlike intrathecally administered neostigmine, does not increase blood pressure,[4] indicating that the systemic effect of neostigmine as a result of intrathecal injection, if any, is unlikely to contribute to the hemodynamic effect. We do not think it is likely, therefore, that the response to neostigmine represents increased synaptic transmission at the sympathetic ganglion, *i.e.*, a systemic effect from the intrathecal injection of neostigmine.

Intrathecal injection of neostigmine produces postoperative analgesia and most commonly is injected at the time of local anesthetic injection for spinal anesthesia. Previous experiments have shown that intrathecal coadministration of neostigmine/bupivacaine or neostigmine/clonidine can diminish hypotension from bupivacaine, an added benefit.[4,5] In the current study, we confirmed the interaction of neostigmine/bupivacaine on the sympathetic nerve activity; however, the precise site of action of local anesthetic agents on sympathetic nerve activity cannot be fully established by our data. Local anesthetic agents injected spinally likely exert their inhibitory effect on the preganglionic nerve axons in the ventral root, the soma of the neurons in the spinal cord, or both. The ability for neostigmine to eliminate the sympathetic suppression of bupivacaine suggests that its action is on the preganglionic neurons in the cord. Another possibility is that the subtotal axonal

block can be compensated for by increased generation of impulses at their origin in the cord by neostigmine.

It has been established that sympathetic responses are not uniform.[23,24] We chose to study the splanchnic nerve activity because splanchnic circulation plays a very important role in regulation of systemic blood pressure.[11,13] Although we have demonstrated that intrathecally administered neostigmine increased whereas intrathecally administered bupivacaine decreased the splanchnic sympathetic outflow, it should not be interpreted that sympathetic activity to all target organs or tissues is altered by these two drugs in a similar fashion. In addition, it should be noted that the anesthetic agents used in this study, including ketamine and lidocaine, are not responsible for the effect of intrathecally administered bupivacaine or neostigmine because the sympathetic nerve activity remained constant for the entire recording period in the saline control group.

We have established a direct and sensitive method to evaluate the influence of spinally administered anesthetic agents on sympathetic nerve activity in a small animal model. Our data, with those from previous studies, suggest that spinal coadministration of neostigmine and bupivacaine represents an effective approach to minimize the alteration of sympathetic outflow and hence blood pressure during spinal anesthesia. One should be cautious, however, in extrapolating the experimental findings to humans. Further clinical investigation is needed to determine the clinical benefit of combined spinal use of neostigmine and bupivacaine.

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