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Intrathecal α2-Adrenergic Agonists Stimulate Acetylcholine and Norepinephrine Release from the Spinal Cord Dorsal Horn in Sheep

An In Vivo Microdialysis Study

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Background: Intrathecal injection of clonidine and dexmedetomidine produce behavioral analgesia by an α2-adrenergic mechanism. Functional and anatomic studies suggest that this analgesia is mediated by cholinergic activation. This hypothesis was directly tested by measuring extracellular acetylcholine concentrations in spinal cord interstitial fluid by means of microdialysis after intrathecal injection of these α2-adrenergic agonists in sheep.

Methods: Twelve sheep with chronically implanted thoracic intrathecal catheters were anesthetized with halothane. Multiple 200-μm-diameter dialysis fibers were inserted surgically at a mid-thoracic level through the dorsal horn and perfused with artificial cerebrospinal fluid. After baseline sampling, either clonidine (100 μg), dexmedetomidine (100 μg), or saline were injected intrathecally. Microdialysis samples were analyzed by high-pressure liquid chromatography for acetylcholine and norepinephrine.

Results: Both α2-adrenergic agonists increased acetylcholine in microdialysate, whereas intrathecal saline had no effect. Analysis of the raw data showed that all groups differed significantly, with greater levels of acetylcholine following administration of dexmedetomidine than clonidine or saline. Unexpectedly, intrathecal clonidine also increased microdialysate norepinephrine levels.

Conclusions: These data are consistent with previous experiments measuring acetylcholine concentrations in cerebrospinal fluid and support analgesia from α2-adrenergic agonists mediated in part by cholinergic activation. In addition, the increase in norepinephrine concentrations after intrathecal administration of clonidine suggest stimulation of norepinephrine release by this agent. (Key words: Analgesia: spinal. Cerebrospinal fluid components: acetylcholine; norepinephrine. Microdialysis. Sympathetic nervous system: α2-adrenergic agonists; clonidine; dexmedetomidine.)

INTRATHECAL injection of the α2-adrenergic agonists clonidine and dexmedetomidine produces behavioral analgesia in animals and humans, although their relative potencies vary among species.[1-4] These agents are thought to produce analgesia by mimicking the action of spinally released norepinephrine from descending noradrenergic inhibitory pathways.[5] Although these agents act by stimulating α2-adrenoceptors, recent evidence suggests that they produce analgesia in part by activating spinal cholinergic neurons. Anatomic studies show a high density of muscarinic and α2-adrenergic ligand binding in superficial areas of the dorsal horn,[6,7] and functional studies in animals show antinociceptive effects of direct agonists at these receptors after intrathecal administration.[8,9] Their interaction is suggested by potentiation of antinociception from intrathecal administration of α2-adrenergic agonists by intrathecal injection of the cholinesterase inhibitor neostigmine[10,11] and inhibition of such antinociception by intrathecal injection of the muscarinic antagonist atropine.[12] Furthermore, intrathecal clonidine administration, in doses producing antinociception in sheep, increases acetylcholine concentrations in cerebrospinal fluid (CSF), an effect that is potentiated by physostigmine and blocked by the α2-adrenergic antagonist idazoxan.[10] In humans, epidurally administered clonidine also increases CSF concentrations of acetylcholine at a time of analgesia.[13] It could be argued, however, that CSF concentrations of neurotransmitters may not

always reflect synaptic release in the spinal cord. One purpose of the current study was to provide more precise localization of the origin of the increase in acetylcholine after intrathecal injection of α_2-adrenergic agonists by sampling from dorsal horn interstitial fluid *via* a microdialysis catheter.

Classically, α_2-adrenergic agonists have been thought to act on presynaptic autoreceptors to reduce the release of norepinephrine, and infusion of α_2-adrenergic agonists decreases circulating norepinephrine concentrations in humans,[14] whereas infusion of an α_2-adrenergic antagonist increases norepinephrine.[15] In the spinal cord, however, most α_2-adrenoceptors are thought to be postsynaptic, because their density in the spinal cord is minimally effected by removal of noradrenergic innervation by chronic spinal cord transection.[16] In contrast to the increase in plasma norepinephrine after peripheral injection of an α_2-adrenergic antagonist, intrathecal injection of idazoxan does not increase CSF norepinephrine and decreases the spinal release of norepinephrine in response to a painful stimulus[17] or to intravenous injection of morphine.[18] A secondary purpose of the current study was to test the effect of intrathecal injection of α_2-adrenergic agonists on dorsal horn microdialysate norepinephrine concentrations.

Methods

Animal Preparation

Microdialysis experiments were performed on 12 ewes of mixed western breeds (weight, 39–52 kg). The animals were fasted for 24 hours and then anesthesia was induced with 5–10 mg/kg ketamine injected intramuscularly. The trachea was intubated and anesthesia maintained with 1% or 2% halothane in oxygen by controlled ventilation. Polyvinyl catheters were inserted into a femoral artery and vein under direct vision and advanced 15 cm centrally. The animal was positioned prone and a small laminotomy was performed at the lumbosacral junction to expose a 1-cm-diameter patch of dura. A 21-gauge polyvinyl catheter was inserted under direct vision through a small nick in the dura and advanced 25 cm cephalad so that its tip was at the mid-thoracic level. All incisions were closed, the catheters were placed in a canvas pouch sewn to the flank, and the ewe was allowed to awaken. At least 3 days passed before the experimental study was begun. During this time, the animals received 900,000 units penicillin G intramuscularly every day.

Surgical Procedure

On the day of the experiment, after a 24-h fast, anesthesia was induced with 5 mg/kg thiopentone sodium injected *via* a femoral vein catheter and maintained with 1% or 2% halothane *via* the endotracheal tube during the surgical preparation. Subsequently, during the microdialysis experiment, anesthesia was maintained with 0.5% halothane in oxygen by controlled ventilation and muscle relaxation with 0.1 mg/kg pancuronium given every 2 h. Ventilation was adjusted to keep the partial pressure of carbon dioxide within a normal range by continuously monitoring end-tidal carbon dioxide levels and intermittently monitoring arterial blood gas tensions. Blood pressure and heart rate were continuously monitored *via* the femoral artery catheter.

The animal was turned prone and bilateral laminotomies were performed on five adjacent interspaces in the mid-thoracic region, leaving the dura and portions of the dorsal spinal processes intact for stability. Six to ten microdialysis probes were inserted transversely in a side-to-side orientation through the superficial dorsal spinal cord at different sites along the exposed cord, with a minimum separation of 1 cm. After completion of the experiment, the location of each probe was determined by visual inspection of sectioned cord. To confirm that the dialysis membrane was functional, some probes were perfused with methylene blue dye, followed by cryosection and microscopic examination.

Microdialysis Procedure

Microdialysis probes were prepared within 3 days of surgical implantation using hollow fiber bundles (Spectrum, Los Angeles, CA) with an internal diameter of 150 μm and a molecular weight cutoff rate of 9,000 Da. The window of active membrane for exchange was precisely defined using two pieces of silica tubing (SGE, Ringwood, Australia) that were inserted through each end of the hollow fiber and advanced so that the tips of each silica tube were separated by 4 mm, corresponding to the length necessary to cover the two dorsal horns of the thoracic spinal cord. The junctions between the silica tubing and the hollow dialysis fiber were sealed using acrylic glue (Borden Inc., Columbus, OH). A wire with a 0.035-mm external diameter (Fisher Scientific, Pittsburgh, PA) was inserted and sealed on one end of the probe and the free end was sharpened, thereby allowing penetration of the probe through the dura mater and cord with minimal tissue damage. After insertion, the portion of the silica tubing connected to

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Table 1. Microdialysate Concentrations of Neurotransmitters prior to Spinal Injection

Treatment Group	Norepinephrine (pmol/ml)	Acetylcholine (pmol/ml)
Saline	11.9 (10.3–13.6)	0.53 (0.19–0.92)
Clonidine	5.6 (0–7.6)*	0.24 (0.15–0.52)
Dexmedetomidine	19.2 (14.5–19.6)	0.47 (0.35–0.66)

Data are median (25th–75th percentiles) of 6–11 values.

* $P < 0.05$ *versus* saline and dexmedetomidine.

the wire was cut and removed to allow perfusion. The inlet of the probe was continuously perfused using a pump at a rate of 2 μl/min at room temperature with artificial CSF composed of 145 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, and 2 mM Na_2HPO_4 in filtered deionized water. During microdialysis, the wound was covered with saline-soaked gauze and surgical towels and a heating lamp was placed over the animal. Spinal cord and animal temperatures, however, were not recorded.

The microdialysate effluent was collected every 15 min into minitubes on ice. The first 90 min of perfusion were considered the washout period, allowing for tissue recovery, and were followed by a 60-min collection (four samples) for baseline. This was followed by intrathecal injection of 100 μg clonidine, 100 μg dexmedetomidine, or saline. All spinal injections were made in a volume of 1 ml followed by a 0.5-ml saline flush. Samples were collected every 15 min for 2 h after spinal drug administration.

Neurochemical Assays. After collection, samples were fast frozen to −20°C and stored at −70°C until assay. Norepinephrine concentrations were determined by high-pressure liquid chromatography with electrochemical detection. This method has an interassay coefficient of variation of less than 9% for norepinephrine and an absolute detection limit of 12 fmol.[19] Acetylcholine concentrations were determined by a different high-pressure liquid chromatography–electrochemical detection method, using equipment other than that for catecholamines. This method has an interassay coefficient of variation of 8% and a detection limit of 50 fmol.[10]

Drugs. Drugs for intrathecal injection were dissolved in sterile 0.9% saline. Dexmedetomidine was a gift from Farmos Pharmaceuticals (Turku, Finland), and clonidine was a gift from Fujisawa, USA (Deerfield, IL). Halothane, ketamine, penicillin G, thiopentone sodium, and meth-

ylene blue were obtained from Barber Veterinary Supply, Richmond, Virginia.

Data Analysis

Data are presented as medians ± 25th and 75th percentiles, because in most cases they were not normally distributed. Within groups, values of acetylcholine and norepinephrine were compared to baseline by one-way Kruskal-Wallis analysis of variance followed by Dunnett's test. The groups were compared for acetylcholine and norepinephrine by two-way nonparametric analysis of variance followed by the Newman-Keuls test. Linear regression analysis was used to examine the relation between concentration of norepinephrine before drug injection and maximum absolute concentration or change in concentration after drug injection. Stepwise logistic regression was used to determine the influence of factors such as drug treatment, probe, and animal on neurotransmitter concentrations. Probability values less than were considered significant.

Results

All animals recovered normally from intrathecal catheter insertion and none exhibited behavioral deficits on the day of the experiment. Arterial blood gas tensions
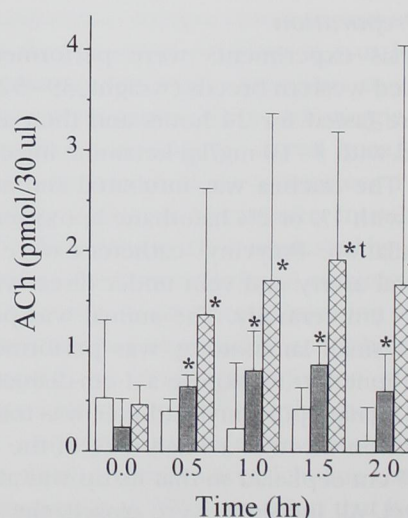
Fig. 1. Spinal cord dorsal horn microdialysate concentration of acetylcholine after intrathecal injection at time 0 of saline (open bars), 100 μg clonidine (filled bars), or 100 μg dexmedetomidine (hatched bars). Each value represents the median plus the 75th percentile of 5–11 values. *$P < 0.05$ compared with baseline; $t < 0.05$ compared with saline.
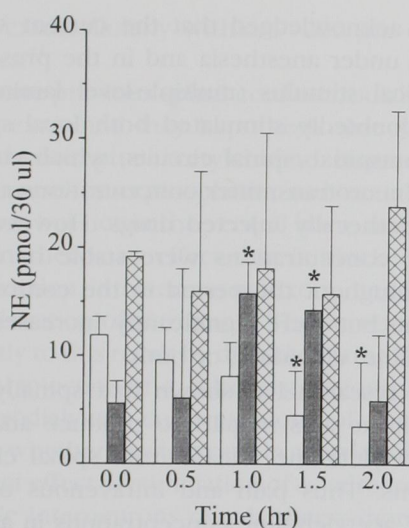
Fig. 2. Spinal cord dorsal horn microdialysate concentration of norepinephrine after intrathecal injection at time 0 of saline (open bars), 100 μg clonidine (filled bars), or 100 μg dexmedetomidine (hatched bars). Each value represents the median plus the 75th percentile of 5–11 values. *P < 0.05 compared with baseline.

and pH and arterial blood pressure remained stable and within normal limits for sheep throughout the experiment in all animals. All probes were located in the dorsal horn. Active dialysis membrane was tested in approximately 20% of all probes, and in every case the membrane was functional, as evidenced by homogeneous diffusion of methylene blue dye into the dorsal horn tissue.

As in previous studies,[18] pairs of microdialysis samples, representing 30 min of collection, were combined to reduce variability. Each probe was considered an independent observation because stepwise logistic regression failed to confirm the individual animal as a significant factor in neurotransmitter concentrations. As a confirmation of this approach, pooling all probe data from each animal and analyzing the results for animals rather than probes did not change the results of the statistical analysis (described here subsequently).

After the washout period, groups did not differ in microdialysate concentrations of acetylcholine (table 1). Dexmedetomidine and clonidine, but not saline, increased acetylcholine in microdialysis samples (fig. 1). The order of magnitude of change in acetylcholine levels after intrathecal injection was dexmedetomidine > clonidine > saline, and the variability in response was greater with dexmedetomidine than with clonidine (fig. 1).

In contrast to acetylcholine, dexmedetomidine failed

to increase microdialysate concentrations of norepinephrine, although clonidine did (fig. 2). This was observed whether pooled data for each animal or individual probes were analyzed independently (fig. 3). The clonidine group had a lower baseline norepinephrine concentration before drug injection than did the other groups (table 1), suggesting that other factors may have contributed to the difference in response to drug. Linear regression analysis failed to support this possibility because there was no significant relation between the baseline norepinephrine level and change in norepinephrine levels after drug treatment. There was, however, a reasonably good correlation (r = 0.68, P < 0.001) between baseline norepinephrine concentration and absolute maximum norepinephrine concentration after drug injection (fig. 4). Note that all but two of the clonidine probes lie above the fitted line for the entire population and all but one of the saline probes lie below it, supporting the statistical difference in responses for these two treatments.

Discussion

Although various studies have indirectly associated spinal noradrenergic–cholinergic interactions in anal-
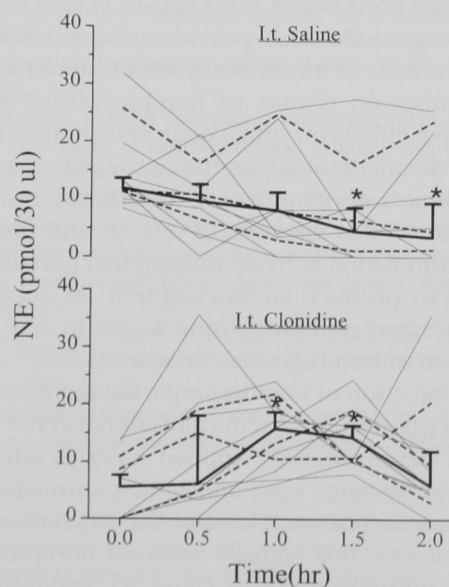

Fig. 3. Effect of intrathecal injection of saline (*upper*) or clonidine (*lower*) on spinal cord dorsal horn microdialysate concentration of norepinephrine in individual probes (thin solid lines), pooled data for each animal (dotted lines), and overall (heavy solid lines with 75th percentiles). *P < 0.05 compared with baseline.
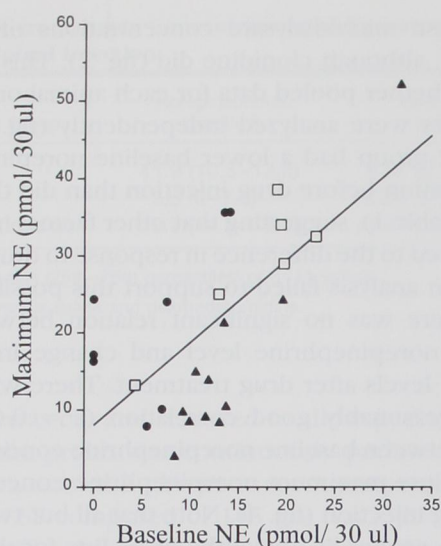
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Fig. 4. Relation between baseline concentration of norepinephrine in spinal cord dorsal horn microdialysate and maximum concentration of norepinephrine after intrathecal injection of saline (filled triangle), 100 μg clonidine (filled circle), or 100 μg dexmedetomidine (open square). There is a significant linear correlation for the entire data set ($P < 0.001$, r = 0.68).

gesia, this is the first study to directly determine, using microdialysis, whether stimulation of α_2-adrenoceptors in the spinal cord dorsal horn causes acetylcholine release. These results also provide unique information about the effects of intrathecally administered α_2-adrenergic agonists on release of norepinephrine and the comparative effects on spinal neurotransmitters of clonidine and dexmedetomidine, the two α_2-adrenergic agonists in clinical development in anesthesia.

Both intravenous injection of opioids and protracted noxious stimulation activate bulbospinal noradrenergic pathways to produce antinociception. As such, these stimuli increase norepinephrine levels in CSF and in dorsal horn microdialysates in animals,[18,20,21] and intrathecal injection of noradrenergic antagonists inhibits antinociception from systemically administered opioids and from heterotopically applied noxious stimuli.[22,23] Similarly in humans, pain and intravenous opioids increase norepinephrine in lumbar CSF.§ Behavioral studies suggest that this spinally released norepinephrine acts at α_2-adrenoceptors to cause its analgesia.[24,25] It

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§ Kimura S, Arai T: The effects of pain and systemically administered opioids on the concentration of noradrenaline (NA) and 5-hydroxyindole acetic acid (5-HIAA) in human CSF (abstract). Proceedings of the 7th World Congress on Pain, 1993.

should be acknowledged that the current study was performed under anesthesia and in the presence of a large surgical stimulus (multiple-level laminotomies), which undoubtedly stimulated both local spinal and spinal – supraspinal – spinal circuits, which could influence local neurotransmitter concentrations and the effect of intrathecally injected drugs. However, neurotransmitter concentrations were stable or decreased slightly throughout the period in the control animals given saline but were significantly increased by dexmedetomidine, clonidine, or both.

Recently research has shown that spinally released norepinephrine acts in part to produce analgesia by causing acetylcholine release from spinal cholinergic interneurons. Thus pain and intravenous opioids increase CSF acetylcholine concentrations in association with the increase in norepinephrine.[17,18] Intrathecal injection of the cholinesterase inhibitor neostigmine produces analgesia, which is enhanced by intravenous opioids and pain,[26,27] in accordance with spinal cholinergic neuronal activation by these stimuli.

Direct epidural or intrathecal injection of α_2-adrenergic agonists in animals and humans also produces a dose-dependent increase in CSF acetylcholine levels.[10,13,28] This increase in CSF acetylcholine is inhibited by intrathecal injection of α_2-adrenergic antagonists and is not mimicked by highly polar α_2-adrenergic agonists that fail to produce analgesia after intrathecal injection in sheep.[10] Although systemic injection of α_2-adrenergic agonists also produces analgesia, these agents are considerably less potent systemically than intraspinally[29] and probably act *via* other mechanisms because epidural, but not intravenous, clonidine increases CSF acetylcholine levels in humans.[30] Corresponding with a cholinergic mechanism of intrathecal α_2-adrenergic agonist analgesia, coadministration of neostigmine further increases CSF acetylcholine and potentiates analgesia from α_2-adrenergic agonists in animals and humans.[28,31]

These previous studies provide considerable evidence that spinally released norepinephrine acts on α_2-adrenoceptors to induce acetylcholine release. However, interpretation of studies that measure neurotransmitters in CSF can be difficult, particularly because cholinesterases are present in CSF and clonidine displays weak inhibition of cholinesterases.[32] Microdialysis has been used in the spinal cord for drug delivery and for acute and long-term sampling of interstitial fluid concentrations of neurotransmitters.[33-35] Although clearly causing some trauma, apparently normal neuronal activity is

recorded extracellularly within 1–200 μm of the dialysis probes.[33]

At least one previous spinal cord microdialysis experiment has examined they hypothesis that norepinephrine α_2-adrenoceptor acetylcholine. Intravenous morphine increases norepinephrine and acetylcholine in dorsal horn, but not in the ventral horn, microdialysate samples in sheep, an effect that is blocked by spinal cord transection, intravenous naloxone, and by intrathecal administration of idazoxan.[18] The current study adds significantly to this result by directly testing the effect of intrathecal injection of α_2-adrenergic agonists on dorsal horn microdialysate concentrations of acetylcholine. However, whether this increase in acetylcholine release was a direct effect of stimulation of α_2-adrenoceptors on cholinergic interneurons or whether a more complex circuit was activated was not addressed in the current study.

Dexmedetomidine and clonidine are approximately equipotent after intrathecal injection in sheep to produce analgesia.[11] In contrast, intrathecal injection of 100 μg (approximately 80% maximum effective dose) of each of these agonists produced quantitatively different effects on microdialysate acetylcholine. Although dexmedetomidine appears more potent than clonidine in increasing levels of acetylcholine in the spinal cord, the difficulty and expense of studying these agents in sheep precluded assessment of dose responses, limiting the certainty of this conclusion. However, both agents clearly increased acetylcholine levels, consistent with other studies of CSF. The time course of the increase in acetylcholine concentrations is consistent with the time course of analgesia from intrathecal injection of this dose of these agents in conscious sheep.[11]

The mechanisms by which intrathecal clonidine increased microdialysate norepinephrine are unclear. Although the animals given clonidine had lower baseline concentrations of norepinephrine, this was unlikely to be the cause of the significant effect in this group only, because there was no significant relation between baseline norepinephrine concentration and change in norepinephrine after intrathecal injection over all groups. Classically, α_2-adrenergic stimulation is thought to reduce norepinephrine release by presynaptic inhibition. However, these data are consistent with two previous studies, which showed that intrathecal injection of the α_2-adrenergic antagonist, idazoxan, decreased rather than increased the effect of a painful stimulus[17] or intravenous injection of morphine[18] to increase CSF norepinephrine. Ongoing studies in our laboratory suggest a

mechanism involving α_2-adrenergic–induced synthesis of nitric oxide in these apparently paradoxical actions of clonidine and idazoxan on norepinephrine concentrations.

Intrathecal injection of the α_2-adrenergic agonists clonidine and dexmedetomidine increases acetylcholine concentrations in microdialysate samples from sheep spinal cord. Clonidine, but not dexmedetomidine, increases norepinephrine concentrations in microdialysate samples, which is consistent with deceases in norepinephrine observed in previous studies after intrathecal injection of an α_2-adrenergic antagonist. These data support a cholinergic, and perhaps noradrenergic, mechanism of analgesia of intrathecally administered α_2-adrenergic agonists.

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