Intrathecal Adenosine Interacts with a Spinal Noradrenergic System to Produce Antinociception in Nerve-injured Rats

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Background: Adenosine analogs produce antinociception in animal models of acute pain, reduce hypersensitivity in models of inflammatory and nerve-injury pain, and stimulate neurotransmitter release in the brain. Adenosine itself is entering clinical trials for analgesia, and the current study examined the effect, mechanisms of action, and interaction with noradrenergic systems of intrathecal adenosine in a rat model of neuropathic pain.

Methods: The left L5 and L6 spinal nerve roots were ligated and, 1 week later, an intrathecal catheter was inserted in male rats. Withdrawal threshold to mechanical stimulation of the left hind paw was determined before and after surgery, confirming mechanical hypersensitivity. The effects of intrathecal adenosine, clonidine, and their combination on withdrawal threshold were determined, and reversal of the effects of adenosine by adenosine and α2-adrenergic antagonists and by destruction of noradrenergic nerve terminals was tested. Finally, spinal cord slices were perfused in vitro with the adenosine agonist 5′-N-ethylcarboxamidoadenosine, and norepinephrine release was measured.

Results: Intrathecal adenosine and clonidine reduced hypersensitivity and interacted in an additive manner. The effects of adenosine were blocked by intrathecal injection of A1 but not A2 adenosine receptor antagonists, by an α2-adrenergic antagonist, and by destruction of spinal noradrenergic nerve terminals. Perfusion of spinal cord slices with 5′-N-ethylcarboxamidoadenosine resulted in a concentration-dependent increase in norepinephrine release.

Conclusion: These data support clinical examination of intrathecal adenosine alone and with clonidine in the treatment of chronic pain states that include a component of mechanical hypersensitivity and suggest that, after nerve injury, adenosine acts to reduce hypersensitivity through spinal norepinephrine release. (Key words: Allodynia; α2-adrenergic; analgesia; neuropathic pain.)

Neuropathic pain is often difficult to manage, and many patients continue to experience severe pain despite optimal use of currently available treatments. A variety of animal models and neurophysiological approaches have focused on an improved understanding of the mechanisms that may underlie the complex phenomenon of neuropathic pain. This study uses a nerve injury model in which ligation of spinal nerves results in primarily mechanical hypersensitivity that is resistant to opioid, but sensitive to α2-adrenergic agonist therapy,
similar to that observed in many patients with chronic neuropathic pain.

Adenosine has been implicated in the stimulation of nociceptors in the periphery but also in the inhibitory modulation of nociceptive information at the spinal level. Adenosine receptors are present on neuronal cell bodies and terminals in the substantia gelatinosa of the spinal cord. Intrathecal administration of adenosine analogs reduces hypersensitivity in animals after peripheral inflammation and nerve injury. There are no such adenosine analogs available for clinical use. However, intravenous infusion of adenosine itself partially alleviates spontaneous pain, allodynia, and hyperalgesia in patients with neuropathic pain. Preclinical neurotoxicity screening has been performed for intrathecal injection of adenosine, and it has been reported to reduce hypersensitivity induced by cutaneous mustard oil application in volunteers. For these reasons, the effects of intrathecal adenosine itself in animal models of hypersensitivity are of interest. We have previously demonstrated that intrathecal adenosine has no effect against noxious heat stimuli in normal rats, but it reduces mechanical hypersensitivity in a postoperative pain model in this species. One purpose of the current study was...
to test the efficacy and determine the potency of intrathecal adenosine to reduce hypersensitivity after nerve injury.

The mechanisms by which adenosine produces analgesia are incompletely understood. Studies in normal animals suggest an action primarily on A1 adenosine subtype receptors in the spinal cord after intrathecal administration. In addition, there is evidence that adenosine agonists produce analgesia, in large part, by interactions with other spinal neurotransmitters, including the two major components of the descending inhibitory systems: serotonin and norepinephrine. A synergistic mechanism involving adenosine, serotonin, and norepinephrine in the spinal cord has been proposed. Finally, adenosine analogs stimulate neurotransmitter release in the brain, and because spinally released norepinephrine produces analgesia by actions on $\alpha_2$-adrenoceptors, it is conceivable that intrathecal adenosine may produce analgesia via spinal noradrenergic activation. Another purpose of this study was to determine the adenosine receptor subtype activated to reduce hypersensitivity, the interaction between adenosine and the $\alpha_2$-adrenergic agonist clonidine, and the reliance of adenosine on noradrenergic mechanisms in reducing hypersensitivity in nerve-ligated animals.

**Methods**

**Surgical Preparation**

The experiments were conducted according to a protocol approved by the Animal Care and Use Committee at Wake Forest University School of Medicine. Male Sprague-Dawley rats (weight, 150–180 g at the time of purchase; Harlan Industries, Indianapolis, IN) were housed separately. They were allowed free access to food and water and were maintained in a 12/12-h day/night cycle. After surgical preparation (described below), they were studied at an average age of approximately 13 weeks and weight of 250–300 g.

Hypersensitivity to mechanical stimulation of the hind paw was induced using the nerve ligation model of Kim and Chung. Animals were anesthetized with halothane (1–2% in oxygen), and the L5 and L6 spinal nerves were exposed on the left side and tightly ligated with silk suture. Sham surgery consisted of surgical exposure of the lateral spinous processes without ligation of spinal nerves. After an 8-day postoperative recovery period, an intrathecal catheter (polyethylene tubing 10) was inserted under halothane anesthesia via an incision in the atlanto-occipital membrane as previously described. The intrathecal catheter was passed 7.5 cm caudally to the level of the lumbar enlargement. Animals with obvious neurologic damage were promptly killed with an overdose of pentobarbital. All experiments were performed 1–2 weeks after intrathecal catheter implantation, and timing of experiments did not differ among experimental groups.

**Mechanical Hyperalgesia Assessment**

Animals were placed in plastic cages on a plastic mesh floor and allowed to acclimate for 30 min. The threshold required to evoke withdrawal of the stimulated paw was tested using calibrated von Frey filaments. The tests were started using a filament that is in the middle of 8 von Frey filaments series with logarithmically incremental stiffness (0.76, 2.65, 5.1, 6.35, 16.7, 28.8, 67.4 g). The filaments were applied to the left paw (ligated-nerve side) in the medioplantar area for about 6 s. The withdrawal thresholds were calculated using the up-down method, as previously described. The method was modified to not include a cutoff of 15 g. All rats were tested twice at a 5-min interval, and the average of these values was used. Each experimental group consisted of five to six rats, and each rat was studied only once.

**Adenosine Action and Interaction with Clonidine**

Intrathecal adenosine ($n = 5$) or saline ($n = 6$) was studied in nerve-ligated animals. Animals received cumulative dosing (doses administered at 30-min intervals based on pilot experiments) with adenosine (cumulative doses of 3, 6, and 20 $\mu$g) or equivalent volumes of saline. To determine the interaction between adenosine and clonidine, two more experimental groups were studied. First, the potency of clonidine was determined with a cumulative dose response of 4, 12, and 20 $\mu$g (doses administered at 30-min intervals based on pilot experiments). Second, a fixed-ratio combination of adenosine and clonidine was administered. Based on analysis of the potency of each drug alone, a 1:1 ratio (by weight) was used, with cumulative dosing of 2, 4, and 8 $\mu$g of the mixture. This therefore consisted of 1 $\mu$g adenosine plus 1 $\mu$g clonidine; 2 $\mu$g adenosine plus 2 $\mu$g clonidine; or 4 $\mu$g adenosine plus 4 $\mu$g clonidine.

Two adenosine antagonists were used. Based on time courses of antagonist action observed in pilot experiments, animals were pretreated with either the A1 receptor–preferring antagonist, 8-cyclopentyl-1,3-dipropylxanthine (9 $\mu$g; $n = 5$), or the A2 receptor–preferring antagonist–preferring...
antagonist, 3,7-dimethyl-1-propargylxanthine (10 μg; n=6), or saline (n = 6) 30 min before administration of 9 μg adenosine. Withdrawal response to application of von Frey filaments was determined before pretreatment, 30 min after pretreatment, and 30 min after adenosine administration. Other animals received vehicle alone without these antagonists (n = 6 in each group). To determine whether the effects of adenosine were mediated by interaction with an α2-adrenergic receptor, we used the α2-adrenergic receptor-selective antagonist, idazoxan (30 μg; n = 6), in the same paradigm. Doses of antagonists were chosen based on previous studies in rats to reverse their specific agonist effects after intrathecal administration.8,20,21

Role of Spinal Noradrenergic System in the Effect of Adenosine

To test whether the antihypersensitivity action of intrathecal adenosine relied on intact noradrenergic terminals, rats were pretreated with an intraperitoneal injection of 10 mg/kg zimeldine (to inhibit uptake of this neurotoxin into serotonergic neurons) 45 min before intraperitoneal injection of the noradrenergic neurotoxin, (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine HCl (DSP-4; 63 mg/kg; n = 6). The effect of intrathecal adenosine was determined 7 days after neurotoxin treatment, a time when spinal cord norepinephrine content is maximally depleted.22 To confirm efficacy of DSP-4 treatment in destruction of noradrenergic terminals, spinal cords from these animals and six other rats that received only intrathecal saline were removed, and norepinephrine was extracted as previously described.23 Briefly, spinal cord was sliced, sonicated on ice, and centrifuged at 16,000 g for 15 min at 4°C. The supernatant was extracted into heptane containing 1% octanol and 0.25% tetraoctylammonium bromide, then back extracted into octanol and 80 mM acetic acid.24 Values were corrected for protein content.25

Norepinephrine was measured by high-pressure liquid chromatography with electrochemical detection.26 High-pressure liquid chromatography was performed using a C18 column Dynamax, 4.6 × 50 mm combined with a 4.6 × 30 guard column (Ranin; Varian Co., Walnut Creek, CA) with a Waters 515 pump to deliver the mobile phase (0.1 M sodium phosphate, 600 mg/l sodium octanesulfonic salt, 5–8% methanol, 1 mM EDTA). In each assay, 50-μl samples were injected through a Ranin AI-1A autosampler and detected by an EC detector (Decade; Antech Leyden Co., Leiden, The Netherlands) at 5-nA range with potential at 620 mV.

Spinal Norepinephrine Release Induced by 5′-N-ethylcarboxamide Adenosine In Vitro

To determine the effect of adenosine receptor stimulation on norepinephrine release in the absence of peripheral or supraspinal effects, a spinal cord slice perfusion system was used. Adult male Sprague-Dawley rats (n = 10 total) that had undergone spinal nerve ligation surgery 2 weeks before and had withdrawal threshold > 2 g on the hind paw ipsilateral to the surgery were killed with sodium pentobarbital (50 mg/kg intraperitoneally), and the spinal cord was removed and placed in ice-cold modified Krebs bicarbonate buffer. The lower lumbar part of spinal cord dorsal horn (corresponding to the level of spinal nerve ligation) was sliced into 0.1–0.5-mm sections manually and quickly loaded into four different superfusion chambers, each on a Grade 1 Whatman filter (10-mm diameter, Whatman International, Maidstone, England), containing 40 mg tissue per chamber. The tissue slices were allowed to equilibrate at 37°C for 30 min while being superfused at a flow rate of 0.45 ml/min with continuously oxygenated (95% O2; 5% CO2) modified Krebs-bicarbonate buffer (pH 7.4) containing 118 mM NaCl, 3.3 mM KCl, 1.2 mM MgSO4, 1.25 mM CaCl2, 1.2 mM KH2PO4, 25 mM NaHCO3, 10 mM Hepes, 0.6 mM ascorbic acid, 11.5 mM glucose, and 10 μM pargyline. After equilibration, three to four 10-min fractions were collected to determine basal norepinephrine concentrations. At the beginning of the fifth fraction, 5′-N-ethylcarboxamide adenosine (NECA) was introduced. Six escalating concentrations of NECA were tested in each of two of the four chambers, with the remaining chambers serving as time controls. NECA concentrations were 10−9 to 10−4 M, in log increments, with three 5-min fractions collected at each concentration. Preliminary analysis showed a plateau of norepinephrine concentrations 10 min after perfusion with NECA; therefore, the last aliquot, representing collection from 10 to 15 min from the last concentration adjustment, was used for each analysis. Samples of each fraction (1.4 ml) were extracted on alumina, using dihydrobenzoic acid as the internal standard. Recovery rates were 35–65%.

Drugs

Drugs used were adenosine (Adenocard; Fujisawa, Deerfield, IL), 8-cyclopentyl-1,3-dipropylxanthine, 3,7-dimethyl-1-propargylxanthine, NECA, zimeldine di-HCl and DSP-4 (RBI, Natick, MA); clonidine HCl, HEPES, methanol, pargyline, sodium phosphate, and idazoxan HCl (Sigma Chemical Co., St Louis, MO); and calcium chloride, l-ascorbic acid, glucose, magnesium sulfate,
potassium phosphate monobasic, potassium chloride, sodium chloride, and sodium bicarbonate (Fisher Scientific Co., Pittsburgh, PA). Adenosine was used in the commercially available solution at a concentration of 3 mg/ml and was diluted with saline as necessary. The adenosine receptor antagonists 8-cyclopentyl-1,3-dipropylxanthine and 3,7-dimethyl-1-propargylxanthine were diluted in dimethyl sulfoxide (DMSO; Sigma Chemical Co.) and 45% 2-hydroxypropyl-β-cyclodextrin (RBI), respectively. All other drugs were diluted in normal saline. Intraperitoneal injections were performed for DSP-4 and zimeldine. Drugs were administered intrathecally in a 5-μl volume followed by 10 μl saline to flush the catheter.

Statistics

Data are presented as median ± 25th and 75th percentile (for raw withdrawal thresholds) or by mean ± SE. Absolute withdrawal thresholds are presented in figure 1 to demonstrate the degree of hypersensitivity developing after spinal nerve ligation. For dose responses of drugs after spinal nerve ligation surgery, withdrawal thresholds were converted to percent of maximum possible effect, which was defined as: 100 × (Postdrug response – predrug response)/(Presurgery threshold – predrug response). Linear regression was used to calculate the dose producing a 50% maximal effect (ED50) for each drug alone and for the fixed-ratio combination. The ED50 was determined for each animal, rather than a probit analysis of the entire data set. Isobolographic analysis was performed as previously described.27 Student t tests was used to compare the difference between the theoretical additive point and the experimentally determined value. The effect of adenosine on withdrawal threshold in sham-treated animals was tested by one-way analysis of variance for repeated measures. The effect of antagonist treatment on percent of maximum possible effect produced by adenosine was tested by one-way analysis of variance followed by Dunnett’s test. The effect of DSP-4 treatment on spinal cord norepinephrine content was tested by a Student t test. The effect of NECA on perfusate norepinephrine concentration was tested by one-way analysis of variance for repeated measures. A P value < 0.05 was considered significant.

Results

Behavioral Experiments

Withdrawal threshold decreased to < 2 g in all animals after spinal nerve ligation but were unaffected by sham surgery. Intrathecal adenosine had no effect on withdrawal threshold in animals after sham surgery. Withdrawal thresholds (median [25th–75th percentiles]) were 58.6 (28.6–58.6) g before adenosine and 46.8 (46.8–58.6) g, 58.6 (46.8–58.6) g, and 58.6 (48.6–58.6) g after 3, 6, and 20 μg adenosine, respectively. In animals with true spinal nerve ligation surgery, intrathecal saline had no effect on withdrawal threshold in animals, but intrathecal adenosine produced a dose-dependent blockade of mechanical hypersensitivity, resulting in return to the presurgery response (fig. 1).

Both clonidine and adenosine produced dose-dependent attenuation of mechanical hypersensitivity after spinal nerve ligation with similar potency (fig. 2; ED50 for clonidine = 4.4 ± 0.7 μg; ED50 for adenosine = 4.8 ± 0.6 μg). Combination of clonidine and adenosine resulted in an additive interaction. This was apparent from inspection of the dose-response curves, which overlay each other (fig. 2), from the ED50 (5.0 ± 1.3 μg) being similar to each drug alone, and from the isobologram (fig. 3).

Neither of the adenosine antagonists or idazoxan or saline altered withdrawal threshold alone (data not shown). The A1-preferring antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DCPX), but not the A2-preferring antagonist, 3,7-dimethyl-1-propargylxanthine, significantly blocked the effect of intrathecal adenosine in nerve-
Ligated animals (fig. 4). Vehicle treatment had no effect (data not shown). Pretreatment with idazoxan also blocked the effect of adenosine (fig. 4). Treatment with the noradrenergic neurotoxin, DSP-4, did not alter withdrawal threshold in nerve-ligated animals (data not shown). As with idazoxan treatment, DSP-4 treatment completely blocked the effect of intrathecal adenosine (fig. 4). Norepinephrine content of lumbar spinal cord tissue was significantly reduced in DSP-4–treated animals (2.2 ± 1.5 ng/mg protein) compared with nerve-ligated animals that received saline treatment (8.4 ± 3.2 ng/mg protein; P < 0.05).

Spinal Cord Slice Perfusion

Norepinephrine concentrations were constant over the time course of the experiment in control slices perfused only with modified Krebs bicarbonate solution (fig. 5). In contrast, inclusion of NECA in the perfusate increased norepinephrine in the perfusate from spinal cord tissue (fig. 5; P < 0.001).

Discussion

Intrathecal adenosine has begun clinical trials in Sweden11,28 and in the United States (studies under National Institutes of Health grant no. GM48085, begun December 1998). Preliminary results suggest enhanced efficacy of intrathecal adenosine in experimentally induced hypersensitivity states, suggesting that this agent may be useful in the treatment of certain chronic pain conditions marked by mechanical hypersensitivity. Indeed, intrathecal injection of an A1-preferring adenosine agonist reduced ongoing pain and allodynia in a patient with such chronic pain.29 Before discussing the current results and the relative potency of adenosine in various animal models of acute and chronic pain, a few characteristics and limitations of the current study and model are included.

Peripheral nerve injury in rats produces marked hypersensitivity to punctate mechanical stimulation, which mimics the human condition in some cases of neuropathic pain. Although the precise mechanisms underlying this pain state are not known, previous reports have indicated important changes that may contribute, including sprouting of large myelinated afferents into the dorsal horn, loss of some dorsal horn interneurons, and
sprouting of sympathetic nerves in the dorsal root ganglia. It has been suggested that spinal nerve ligation–induced hypersensitivity involves a local sympathetic nervous system component related to sprouting of sympathetic fibers in the dorsal root ganglia, although other investigators have failed to observe a sympathetic component, which may be variable and rat strain-dependent.

The potency of drugs to alleviate mechanical hypersensitivity after spinal nerve ligation depends on the definition of normal. In the current study, we used the presurgery withdrawal threshold, calculating a 100% effect as that which would return the threshold to this value. However, at least 2 and as much as 3 weeks passed from the time of surgery until drug testing was performed to allow establishment of stable hypersensitivity, then to allow recovery time from the spinal catheterization. It is possible that withdrawal threshold could have increased during growth in normal animals over this time, as evidenced by the higher withdrawal thresholds in the sham-treated animals. Had we used this age-matched control as “normal,” the apparent potency of drugs studied would have been less. Other investigators have used an arbitrary cutoff, typically 15 g, which is considerably less than our presurgery threshold, and would make drugs seem more potent. Still other investigators have used the contralateral side as a control, but bilateral changes in the spinal cord make this inappropriate. Thus, although one can compare one drug to another within a model definition, it is difficult to compare studies with different definitions of 100% effect and to hazard an extrapolation about potency to treat human pain. Similarly, although the potency of drugs may be different in response to suprathreshold stimulation than in the current method, with most stimulation occurring around the threshold, it is the abnormal threshold that is the target of treatment in the clinical setting of chronic pain.

The current study with adenosine itself supports previous observations with synthetic adenosine analogs, which demonstrate efficacy to reduce thermal hypersensitivity after peripheral inflammation and mechanical hypersensitivity after spinal cord injury and nerve injury. Antagonist studies are also consistent with the suggestion that this antihypersensitivity action is caused by stimulation of A1 adenosine receptors. It is unlikely that nonspecific effects of the vehicles used explained the selective blockade by the A1 antagonist, because vehicles alone had no effect. A small A2 component in reducing hypersensitivity cannot be excluded in the current study with small numbers of animals. Adenosine

![Graph](image-url)

**Fig. 4.** Effect of pretreatment on the effect of 9 µg intrathecal adenosine in animals after spinal nerve ligation. Animals were pretreated with saline and then received adenosine, or were pretreated with the A1-preferring adenosine antagonist, DCPX, the A2-preferring adenosine antagonist, 3,7-dimethyl-1-propargylxanthine, the α2-adrenergic antagonist, idazoxan, or the noradrenergic neurotoxin, DSP-4. Response is depicted as percent maximum possible effect (%MPE), defined as return of withdrawal threshold to presurgery levels. Values are mean ± SE of five to six animals. *P < 0.05 compared with saline pretreatment.

![Graph](image-url)

**Fig. 5.** Concentration of norepinephrine in perfusates of spinal cord slices from nerve-ligated animals with continuous perfusion with modified Krebs-bicarbonate solution (circles) or with the A1 and A2, nonselective adenosine agonist, 5'-N-ethylcarboxamidoadenosine (NECA). Values are mean ± SE of 8 to 12 experiments. *P < 0.05 compared with 0 control.
itself has been the subject of few investigations. Intrathecal adenosine did not affect pain from local heat application to the skin but did reduce mechanical hypersensitivity from mustard oil application in human volunteers.11 Similarly, we observed lack of effect from intrathecal adenosine in normal rats to heat stimulation of the hind paw but found its efficacy against mechanical hypersensitivity after skin incision.12 It seems that intrathecal adenosine is considerably more potent against nerve injury–induced mechanical hypersensitivity (ED$_{50}$ = 4.8 ± 0.6 μg from current study) than against postoperative mechanical hypersensitivity (ED$_{50}$ = 154 ± 22 μg).12 It would seem that the potency of intrathecal adenosine (nerve injury > following skin incision >> normal animal) reflects an increased expression of purinergic inhibitory mechanisms in hypersensitivity states. Thus, one might expect to observe greater efficacy from a fixed dose of intrathecal adenosine in patients with chronic pain and mechanical hypersensitivity than in those with postoperative pain.

Interactions between intrathecal adenosine and clonidine are of interest for practical and mechanistic reasons. Clonidine is effective in nerve injury–induced hypersensitivity4 and is approved for treatment of chronic neuropathic pain. Because clonidine therapy can be limited by sedation and hypotension in some patients with chronic pain,37 the current demonstration of enhancement of clonidine’s effect by adenosine suggests that clonidine dose, and perhaps these side effects, could be reduced by addition of adenosine. We did not measure blood pressure in the current study, thus we cannot exclude the possibility that adenosine has no effect or even worsens clonidine-induced hypotension.

The additive interaction between clonidine and adenosine observed in the current study is consistent with, although it does not prove, a common final pathway for effect. A similar mechanism of action for adenosine and clonidine is also suggested because of a similar increase in potency of clonidine in nerve-injured animals (ED$_{50}$ = 4.4 ± 0.7 μg, current study) compared with the postoperative model (ED$_{50}$ = 51 ± 16 μg).12 We have proposed that adenosine may act via stimulation of spinal norepinephrine release because intrathecal phenolamine blocked the antihypersensitivity effects of intrathecal adenosine in the postoperative rat model.12 Furthermore, we observed in the current study that the antihypersensitivity effect of intrathecal adenosine was blocked by the specific α₂-adrenergic antagonist idazoxan. Destruction of noradrenergic nerve terminals, demonstrated to be reasonably complete by decrease in spinal cord norepinephrine content, also abolished the effect of adenosine. Finally, adenosine agonist-induced stimulation of norepinephrine release in vitro suggests that spinally administered adenosine may act to reduce hypersensitivity by activation of local, spinal noradrenergic terminals to release norepinephrine, rather than by some other peripheral or supraspinal activation of noradrenergic pathways. Further studies are necessary to determine the mechanisms by which adenosine stimulates norepinephrine release and the conditions under which this occurs.

In summary, intrathecal adenosine inhibits mechanical hypersensitivity induced by spinal nerve ligation in rats and does so with an apparent potency > 10-fold greater than that necessary to inhibit mechanical hypersensitivity after surgical incision. Intrathecal adenosine produces this inhibition by an action on α1 adenosine receptors and by interaction with α₂-adrenergic receptors, probably by stimulation of norepinephrine release in the spinal cord. These data support clinical trials of intrathecal adenosine alone and in combination with clonidine in the treatment of chronic pain with hypersensitivity.

References

SPINAL ADENOSINE IN NEUROPATHIC PAIN


