Intraspinal Adenosine Induces Spinal Cord Norepinephrine Release in Spinal Nerve-ligated Rats but not in Normal or Sham Controls

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Background: Intrathecal adenosine is antinociceptive under conditions of central sensitization, but not in response to acute stimuli in normals. The reasons for this selective circumstance of action remain unclear, but some evidence links adenosine's antinociceptive effects to release of norepinephrine by terminals in the spinal cord. The purpose of this study was to test whether spinal adenosine induces norepinephrine release selectively in settings of hypersensitivity.

Methods: Rats randomly assigned to spinal nerve ligation, sham operation, or no operation were anesthetized. A microdialysis fiber was implanted in the spinal cord dorsal horn at the L5–L6 level and perfused with artificial cerebrospinal fluid. After washout and a baseline sample period, adenosine at various concentrations was infused through the fiber for 150 min, and samples were collected every 15 min.

Results: In ligated, but not in sham or normal animals, adenosine perfusion increased norepinephrine in spinal cord microdialysates in a concentration-dependent manner. The effects of adenosine plateaued after 75 min and remained stable until the end of the experiment. Intravenous injection of selective adenosine A1 and A2 receptor antagonists revealed that adenosine's effect on spinal norepinephrine release was A1 receptor mediated.

Conclusions: This is the first study to provide direct evidence that adenosine is able to release norepinephrine in spinal cord dorsal horns in living animals. However, this effect was only seen in animals after spinal nerve ligation. These data are consistent with behavioral studies demonstrating that adenosine's antinociceptive effects in rats after spinal nerve ligation is totally dependent on intact spinal noradrenergic terminals and can be blocked by spinal α2-adrenergic receptor antagonists.

RESEARCH over the past decade has introduced the endogenous nucleoside adenosine as a novel drug for the treatment of pain. Adenosine's effect is more consistently demonstrated in chronic pain rather than acute postoperative pain.[1–4] Particularly striking are the long-lasting (more than 24 h) antinociception in both animal and human studies produced by intrathecally administered adenosine, and its lack of efficacy to acute noxious stimuli in normals compared to its efficacy in animals or humans with chronic pain.[3,4]

The reasons for this selective action of adenosine in the setting of chronic pain remain obscure. The hypothesis that differences in pharmacokinetics in cerebrospinal fluid (CSF) could underlie the selective action was disproved by a microdialysis study in rats and repeated CSF analyses in humans.[4,5] Because it is widely accepted that adenosine mediates its antinociceptive effects *via* binding to A1 receptors with consecutive activation of Gi-proteins, it was further suggested that the process of central sensitization could have altered either adenosine receptor number or their ability to activate G-proteins in the spinal cord. These hypotheses have been shown to be unlikely, because a radioligand binding study revealed no changes in dorsal horn A1 receptor number between normal and spinal nerve-ligated (SNL) rats.[5] Furthermore the maximum amount of G-protein activation in $[^{35}S]GTP\gamma S$ spinal cord autoradiography between control and SNL animals also remained unchanged.[6]

These studies indicate that direct changes in the spinal cord adenosine system due to the mechanisms of central sensitization seem not to underlie adenosine's antinociceptive effect. On the other hand, recent studies indicate a dependence of antinociception mediated by adenosine on the spinal cord noradrenergic system. For example, adenosine and the α_2-adrenergic receptor agonist clonidine, which mimics the effect of norepinephrine, interact in an additive rather than synergistic manner, consistent with a serial action.[7] In the same study the antinociceptive effect of adenosine was blocked by co-administration of the α_2-receptor antagonist idazoxan.[7] Finally, destruction of spinal noradrenergic terminals with neurotoxins abolishes the antihypersensitivity effects of adenosine and adenosine modulators.[8]

These results led to the hypothesis that spinal adenosine exerts its antinociceptive effects under conditions of central sensitization by inducing the release of norepinephrine in the spinal cord dorsal horn. To test this hypothesis, we performed a microdialysis study in living animals with and without hypersensitivity.

Materials and Methods

All animal studies were conducted at Wake Forest School of Medicine and were approved by the Animal Care and Use Committee. Male rats (Harlan Sprague-Dawley) weighing 200–300 g were randomly assigned to SNL, sham operation, or no surgery. Animals were

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housed at 22°C and under a 12-h light/dark cycle, with free access to food and water.

SNL and Sham Operation

SNL was performed as previously described.[9] In brief, halothane anesthesia was administered and the left L5 and L6 spinal nerves were isolated adjacent to the vertebral column in both SNL and sham groups. In the SNL group, L5 and L6 spinal nerves were tightly ligated with 6–0 silk sutures distal to the dorsal root ganglion.

After a recovery period of 13 days in all groups, left paw tactile hypersensitivity was confirmed by measuring hind paw withdrawal threshold to von Frey filaments, using a previously described up–down method.[10] Only rats without signs of neurologic impairment were included for study. In the SNL group, only animals with a withdrawal threshold below 4 g were included.

Microdialysis Fiber Preparation and Implantation

A microdialysis fiber (200 μm outside diameter; 45,000 molecular weight cut-off) was made impermeable by epoxy coating, except for a 2-mm gap to traverse the spinal cord dorsal horns. Preliminary experiments revealed an efficiency of recovery of 4% at a flow rate of 2 μl/min.

Rats were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital. The right jugular vein was catheterized and anesthesia maintained by a continuous intravenous infusion of pentobarbital. Heart rate and mean arterial blood pressure were continuously monitored *via* a catheter implanted into the right carotid artery. A heating blanket was used to maintain rectal temperature between 36° and 37°C.

The microdialysis fiber was inserted to traverse both dorsal horns of the spinal cord as previously described.[11,12] In brief, after removal of skin and muscle over the T13 vertebra, two holes were drilled in the lateral laminae to expose a portion of the lumbar spinal cord (L5–L6). The dialysis fiber was passed through the holes and the dorsal spinal cord and stabilized with dental cement applied to the bone. The fiber was connected to a microvolume infusion pump at its inflow side *via* 20 cm of a polyethylene-20 catheter, and the outflow side was connected to 6 cm of polyethylene tubing.

After fiber implantation the system was perfused with artificial CSF (concentration in mM: Na^+ 151.1, K^+ 2.6, Mg^{2+} 0.9, Ca^{2+} 1.3, Cl^- 122.7, HCO_3^- 21, HPO_4 2.5, and dextrose 3.5) for a 90-min washout period and an additional 15-min period for baseline norepinephrine determination. During the following 150 min, artificial CSF or various concentrations of adenosine were perfused through the fiber and samples were collected every 15 min. At the end of this period, 10 mM nicotine was perfused in some experiments as a positive control to demonstrate the ability to measure drug-induced increases in norepinephrine in microdialysates. Throughout the entire experiment the perfusion rate was set

2 μl/min. All samples were collected on ice and stored at −80°C until analysis. Finally, the fibers were perfused for 15 min with methylene blue to stain the areas surrounding the active dialysate window in the spinal cord. The animals were then killed by pentobarbital injection, and the spinal cords were removed and postfixed with 8% buffered formalin. After sectioning, the placement of the dialysis fibers was verified microscopically.

Norepinephrine Analysis

Each microdialysis sample was extracted on alumina, using dihydrobenzoic acid as the internal standard. Recovery rates were 35–65%. Norepinephrine was measured by high-pressure liquid chromatography with electrochemical detection as previously described.[13] The detection limit for norepinephrine using this system is 0.2 pg/20-μl sample.

Experimental Groups

In the first phase of the study, SNL (n = 5), sham-operated (n = 5), and normal (n = 4) animals received 5 mM adenosine by continuous microdialysis fiber perfusion for 150 min. The commercial preparation of adenosine (Fujisawa Pharmaceuticals, Deerfield, IL) was diluted in artificial CSF. Because a significant norepinephrine release was detected only in the SNL group, different concentrations of adenosine (0.5 mM, n = 4; 1 mM, n = 6) and artificial CSF (n = 4) were subsequently perfused in SNL animals only. To verify that the effect of adenosine on norepinephrine release was due to an adenosine receptor-mediated process, animals were treated with the adenosine A1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; n = 4), or the adenosine A2 receptor antagonist 3,7-dimethyl-1-propargylxanthine (n = 4), administered intravenously in a dose of 1 mg/kg 10 min before the start of the 5-mM adenosine perfusion.

Statistical Analysis

Data are presented as mean ± SE or median ± 25th and 75th percentiles, as appropriate. Because of the great interindividual variability of baseline norepinephrine concentrations, all microdialysis data are shown as percent change from baseline. Statistical analysis was performed using a one- or two-way repeated measures analysis of variance followed by the Dunnett test, or Kruskal-Wallis one-way analysis of variance on ranks followed by the Dunn test. $P < 0.05$ was considered significant.

Results

Baseline microdialysate norepinephrine concentrations did not differ among groups (table 1). Continuous perfusion of spinal cord dorsal horns with 5 mM adenosine led to an increase in microdialysate norepinephrine

Table 1. Baseline Norepinephrine Concentrations

Group	Concentration (pg/50 μl)
Spinal nerve ligation	
Adenosine, 5 mM	12.86 ± 3.43
Adenosine, 1 mM	12.35 ± 3.23
Adenosine, 0.5 mM	8.05 ± 1.58
ACSF	8.22 ± 0.72
Adenosine, 5 mM + DPCPX	6.57 ± 0.90
Adenosine, 5 mM + DMPX	8.09 ± 1.34
Control	
Normal-adenosine, 5 mM	16.46 ± 2.40
Sham-adenosine, 5 mM	15.28 ± 4.31

Data are mean ± SE. One-way analysis of variance revealed no differences among groups.

ACSF = artificial cerebrospinal fluid; DPCPX = 1,3-dipropyl-8-cyclopentyl-xanthine; DMPX = 3,7-dimethyl-1-propargylxanthine.

concentrations only in the SNL group (fig. 1). Norepinephrine concentrations in the SNL group peaked 75 min after the beginning of infusion, and remained stable thereafter. Microdialysate norepinephrine concentrations in the SNL group were significantly greater than sham or normal controls from 30 min onwards after the start of dorsal horn perfusion with adenosine (fig. 1). To test whether adenosine's failure to increase norepinephrine concentrations in control groups was due to deterioration of the preparation over time, microdialysis fibers were perfused with 10 mM nicotine at the end of the experiment. Nicotine was chosen because it was known to stimulate norepinephrine release by an adenosine-independent mechanism.[14] In controls, nicotine was able to increase microdialysate norepinephrine concentrations to 321 ± 2% of baseline (data not shown; $P <$ 0.05), indicating viability of the preparation.
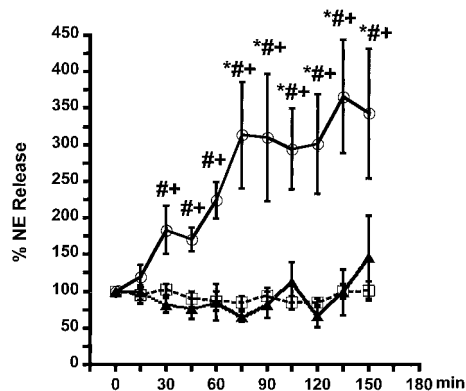

Fig. 1. Time–effect curve of microdialysis fiber perfusion with 5 mM adenosine on dorsal horn microdialysate norepinephrine (NE) concentrations in spinal nerve-ligated (SNL; *circles*), sham-operated (*triangles*), and normal (*squares*) animals. Adenosine perfusion started after a 15-min baseline-sampling period at time-point 0 min and ended after 150 min. Data represent percent change from baseline for each group, respectively, and are shown as mean ± SE. * *P* < 0.05 within a group compared to baseline; # *P* < 0.05 compared to sham controls; + *P* < 0.05 compared to normal controls.
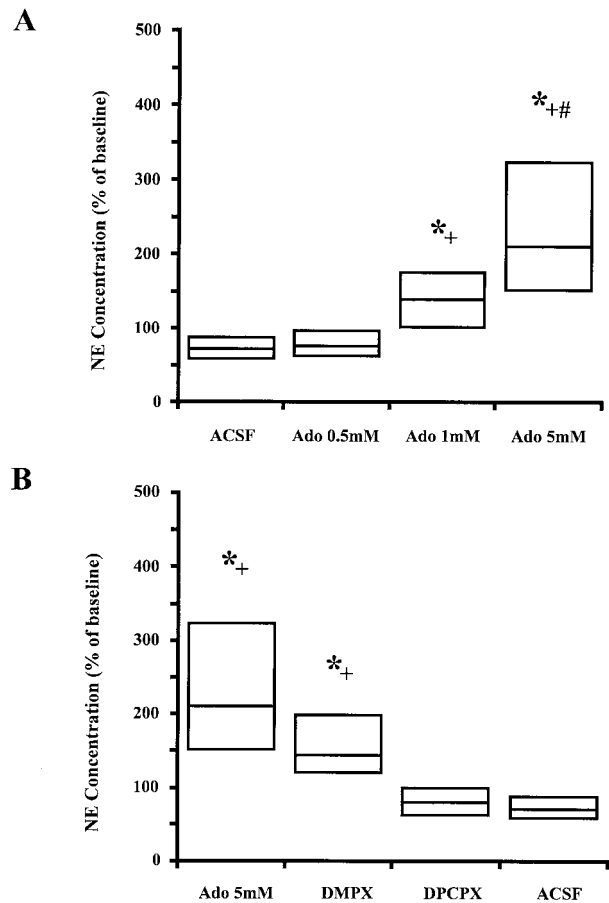

Fig. 2. (*A*) Concentration–response relationship between intraspinal microdialysis perfusion with adenosine (Ado) or artificial CSF and dorsal horn microdialysate norepinephrine (NE) concentrations in animals with spinal nerve ligation. Data are presented as median ± 25th and 75th percentiles. * *P* < 0.05 compared to the artificial CSF group; + *P* < 0.05 compared to 0.5 mM Ado group; # *P* < 0.05 compared to 1 mM Ado group. (*B*) Effect of intravenous treatment of SNL animals with the A1 receptor-specific antagonist, DPCPX (1,3-dipropyl-8-cyclopentylxanthine), or the A2 receptor-preferring antagonist, DMPX (3,7-dimethyl-1-propargylxanthine), on the increase in microdialysate norepinephrine concentrations induced by 5 mM adenosine perfused in the catheter. Data are presented as median ± 25th and 75th percentiles. * *P* < 0.05 compared to the artificial CSF group; + *P* < 0.05 compared to DPCPX group.

When all postadenosine microdialysate observations over time were averaged, there was a concentration–response relationship for adenosine's effect on change in norepinephrine concentration (fig. 2, *A*).

Intravenous injection of adenosine receptor antagonists clearly indicated the receptor subtype involved in the increase in norepinephrine concentrations. The specific A1 adenosine receptor antagonist, DPCPX, but not the adenosine A2 receptor-preferring antagonist, 3,7-dimethyl-1-propargylxanthine, significantly inhibited the increase in microdialysate norepinephrine concentrations induced by 5 mM adenosine (fig. 2, *B*). Nicotine (10 mM), applied to the DPCPX group at the end of the experiment, resulted in an increase in microdialysate
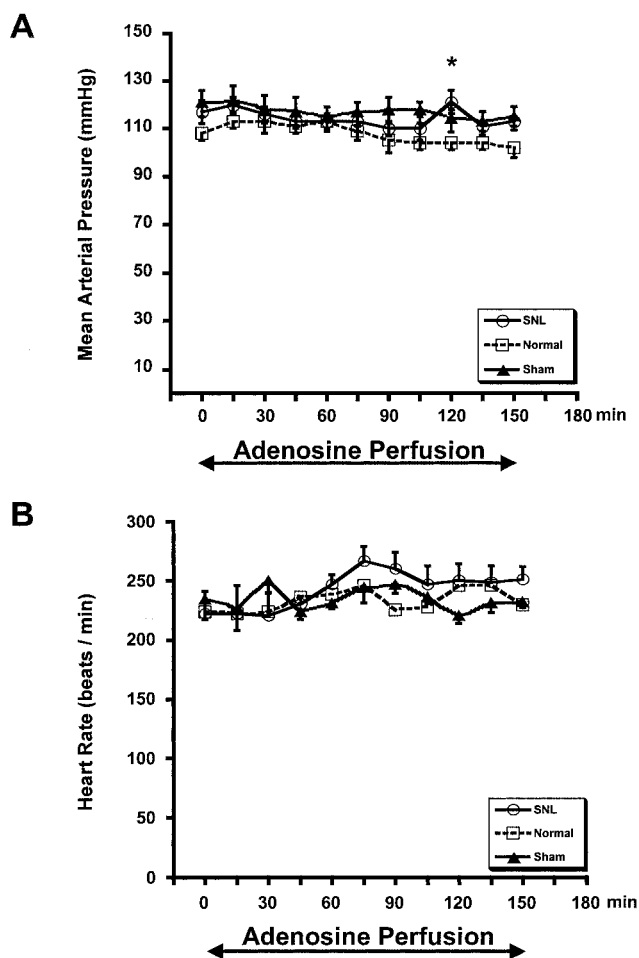
Fig. 3. Time–effect curve of 5 mM intraspinal adenosine perfusion by microdialysis on mean arterial pressure (*A*) or heart rate (*B*) in spinal nerve-ligated (SNL; *circles*), sham-operated (*triangles*), and normal (*squares*) animals. Data are presented as mean ± SE. *P < 0.05 compared to normal controls.

norepinephrine concentrations of 197% above baseline, indicating viability of the preparation (data not shown).

Adenosine perfusion in the microdialysis fibers had no effect on hemodynamic variables. Hence, mean arterial blood pressure (fig. 3, *A*) and heart rate (fig. 3, *B*) remained stable throughout the experiment without any significant change from baseline in any group.

Discussion

Several previous indirect observations have suggested that the selective efficacy of intrathecal adenosine, which is only present in settings of hypersensitivity, reflects a spinal circuit that involves the release of norepinephrine and actions on α_2-adrenoceptors. The major contribution of the current study is the direct assessment of spinal norepinephrine release by locally administered adenosine, and the results indicate that spinal nerve ligation is associated with a remarkable plasticity, leading to the capability of adenosine to release norepinephrine.

Microdialysis sampling of interstitial fluid is often used to gauge neurotransmitter release from the central nervous system and from peripheral tissues. Several caveats should be considered in interpretation of these types of studies. First, one is measuring interstitial fluid concentrations, which may reflect spillover from synaptic release, in this case of norepinephrine, but could also reflect tissue trauma or reaction to the chemicals associated with the microdialysis fiber surface. For this reason, a washout period is incorporated in the study design, and changes from a stable baseline period are interpreted as due to experimental manipulations rather than these factors. Second, there is often a large variability from one experiment to another with this method, reflecting differences in efficiency of dialysate transfer with each probe, differences in local trauma and hemorrhage surrounding the probe, and differences in probe location relative to the synapses of interest, as well as other factors. For this reason, change from baseline is often used, rather than absolute neurotransmitter concentrations. We did not attempt in the current study to determine absolute norepinephrine concentration in the interstitial fluid, which would have required a reverse dialysis method. However, we did control for catheter location, which was in each case covering the superficial dorsal horns bilaterally. Third, secondary effects of drugs administered can result in synaptic release unrelated to direct actions. Although anesthesia could alter spinal norepinephrine release, this was maintained constant in the current study. Similarly, adenosine did not alter blood pressure or heart rate, which could have reflected changes in sympathetic nervous system control regulated by spinal norepinephrine.

On the other hand, several observations suggest that the increase in norepinephrine observed in SNL animals in the current study was directly related to local action of adenosine on spinal A1 receptors. Adenosine perfusion increased microdialysate norepinephrine concentrations in a concentration-dependent manner. This effect of adenosine was reversed by adenosine receptor antagonists, specifically by A1 but not A2 antagonists, consistent with behavioral studies indicating that intrathecal adenosine reverses hypersensitivity in SNL animals by actions on A1 adenosine receptors.[7,15,16] The current results are in accordance with recent studies showing the ability of synthetic adenosine analogs to stimulate norepinephrine release in spinal cord slices of SNL animals.[7] They are also consistent with the loss of intrathecal adenosine activity in SNL animals seen after the spinal cord noradrenergic system is destroyed.[8]

Mechanisms of Adenosine-induced Norepinephrine Release

The mechanisms involved in adenosine's action on spinal norepinephrine release under conditions of cen-

tral sensitization remain unknown. It is conceivable that the pathophysiologic process induced by nerve injury leads to neuroanatomical changes in the spinal cord either with an increased expression of adenosine A1 receptors or with a change in the number of noradrenergic fibers in the spinal cord. The former seems unlikely because spinal cord A1 receptor number, as measured by radioligand binding, does not differ between dorsal horns of SNL and normal control animals.[5] Similarly, although it is possible that G-protein coupling efficiency in response to A1 adenosine receptor stimulation could be increased after SNL, this hypothesis was not supported in a recent study.[6] The latter may be possible, because SNL induces a diffuse increase in noradrenergic fiber density in the dorsal horn ipsilateral to nerve injury (Weiya Ma, Ph.D., personal communication, Assistant Professor, Department of Anesthesiology, Wake Forest University School of Medicine, June, 2002).

These explanations for adenosine's ability to induce norepinephrine release after SNL reflect neuroanatomical or membrane-bound extracellular mechanisms. However, the underlying changes for the observed adenosine effects might also be found at the level of intracellular signal transduction cascades. G-proteins are composed of three subunits, α and $\beta\gamma$. Although the main research emphasis over the last decades has been on the physiologic functions of the α subunit, a growing body of evidence attributes major second messenger pathways to $\beta\gamma$ subunit-mediated actions. These subunits are capable of inducing phospholipase C and consecutively downstream protein kinase C activity. Even though to date the effects of protein kinase C have been mostly attributed to excitatory pronociceptive pathways *via* the isoenzyme,[17] there is also evidence that protein kinase C isoforms may mediate norepinephrine release in inhibitory pathways.[18] Indeed, norepinephrine release in cortex is induced in some circumstances by mechanisms involving protein kinase C activation.[19]

Implications on Adenosine's Antinociceptive Properties

Regardless of the mechanisms by which adenosine activates spinal norepinephrine release after SNL, this selective action, as indicated directly in the current study and indirectly in previous ones, opens a new perspective in the understanding of adenosine's antinociceptive effects under circumstances of central sensitization. Epidural clonidine is approved for the treatment of neuropathic cancer pain, and efficacy of α_2-adrenoceptor agonists for analgesia increases after neuropathic injury.[20,21] Yet, clinical utility of clonidine is limited by adverse effects from redistribution of this drug to supraspinal sites, causing sedation, hypotension, and dry mouth. The exciting observation from the current study

that local adenosine stimulates norepinephrine release only in spinal cords of SNL animals suggests that intrathecal adenosine may be capable of producing a highly localized α_2-adrenoceptor stimulation by local release of norepinephrine without these adverse effects. Indeed, preliminary studies indicate that intrathecal adenosine produces pain relief in patients with neuropathic pain without causing hypotension or sedation.[1]

Conclusion

In summary, perfusion of the spinal cord dorsal horn with adenosine *via* an implanted microdialysis probe results in concentration-dependent increases in norepinephrine in microdialysates in SNL, but not normal and sham-operated animals. A1, but not A2, adenosine receptor-specific antagonists block this effect. These data agree with previous behavioral and *in vitro* studies indicating that nerve injury results in a new direct or indirect mechanism by which spinal adenosine A1 receptor activation induces dorsal horn norepinephrine release, ultimately leading to analgesia by an α_2-adrenoceptor mechanism. These data further support ongoing studies of intrathecal adenosine for the treatment of neuropathic pain.

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