Morphine-induced Spinal Release of Adenosine Is Reduced in Neuropathic Rats

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Background: Spinally administered opioids show decreased potency and efficacy in the treatment of neuropathic pain. As reported previously, morphine stimulates spinal opioid receptors to effect adenosine release, which acts at adenosine receptors to produce analgesia. The authors hypothesized that morphine induces less adenosine release in neuropathic compared with normal rats, explaining its reduced potency and efficacy.

Methods: Sprague-Dawley rats (200–250 g) were divided into three groups: no surgery (n = 52), sham surgery (n = 20), or left L5 and L6 spinal nerve ligation (n = 64). Two weeks after surgery, mechanical hypersensitivity of the left hind paw was verified. For each experiment, a crude synaptosomal P2 suspension was prepared by homogenizing cervical and lumbar dorsal spinal cord halves from four rats, followed by differential centrifugation, and aliquots incubated with morphine sulfate from 10^-8 to 10^-3 M alone or in presence of 10^-3 M dipyridamole. Extrasympatonic concentrations of adenosine were analyzed by high-pressure liquid chromatography.

Results: Synaptosomal release of adenosine in the absence of morphine was similar between groups. Morphine produced a concentration-dependent adenosine release, which was less in synaptosomes from dorsal lumbar spinal cord in spinal nerve ligation compared with normal or sham animals. This reduction was removed by adding dipyridamole.

Conclusion: Morphine normally stimulates spinal release of adenosine, a potent antihypersensitivity compound. Because this effect of morphine is diminished in spinal nerve ligation animals, one explanation for decreased efficacy and potency of opioids in the treatment of neuropathic pain may be a dipyridamole-sensitive disruption in the opioid–adenosine link in the spinal cord.

Although spinally administered opioids are effective in the treatment of cancer and somatic pain, they show decreased potency and efficacy in the treatment of neuropathic pain. The mechanisms for this decrease in efficacy and potency remain unclear. The current study examines an opioid–adenosine interaction as a potential explanation.

Several studies support the hypothesis that adenosine is involved in opioid-induced antinociception. Systemic application of the adenosine receptor antagonist amiphylline inhibits the antinociceptive effect of systemic morphine. Similarly, intrathecal injection of other methylxanthine adenosine antagonists inhibits antinociception produced by intrathecal morphine in both hot-plate and tail-flick tests in mice and rats. Morphine releases adenosine into intrathecal space superfusates from the intact spinal cord by a naltrexone-sensitive mechanism. In biochemical experiments, morphine produces a dose-dependent, opioid receptor-mediated release of adenosine in synaptosomes from the dorsal but not from the ventral half of the spinal cord. This release occurs via nucleoside transporters that are sensitive to the inhibitor dipyridamole but insensitive to nitrobenzylthioinosine. Therefore, dipyridamole is usually chosen instead of nitrobenzylthioinosine as the nucleoside transport inhibitor for adenosine release or re-uptake studies.

Antinociceptive doses of morphine in normal rats are less effective in suppressing tactile allodynia after injury to spinal nerves, such as tight ligation of the L5 and L6 spinal nerves (SNL). In contrast, spinally administered adenosine analogs or adenosine kinase inhibitors produce antinociception in normal animals and remain effective to reduce allodynia and hyperalgesia after inflammation and nerve injury. In a recent study, efficacy of spinally administered morphine to reduce tactile allodynia in rats after SNL was restored by intrathecal coapplication of the adenosine re-uptake inhibitor dipyridamole.

These data are consistent with a reduced efficacy of intrathecal morphine in nerve injury–induced tactile hypersensitivity, which can be reversed by adenosine re-uptake inhibition by dipyridamole, indicating a potential disruption in the normal morphine–adenosine circuit. This disruption would be expected to be segmentally restricted to areas near the site of injury in the lumbar spinal cord. To test these hypotheses, we investigated the effect of SNL on morphine-induced release of adenosine in synaptosomes near (lumbar) and far (cervical) from the site of spinal nerve injury, in the presence or absence of dipyridamole.

Materials and Methods

Animals and Surgery
After obtaining approval from the Animal Care and Use Committee (Wake Forest University School of Medicine, Winston-Salem, NC), 136 male Sprague-Dawley rats (200–250 g at the time of purchase; Harlan Industries, Indianapolis, IN) were studied. They were housed separately and were allowed free access to food and tap water and were maintained in a 12-h day/12-h night

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cycle. Animals were separated into three groups: no surgery (n = 52), sham surgery (n = 20), or lumbar SNL (n = 64), which was used to induce hypersensitivity to mechanical stimulation of the left hind paw. Animals were anesthetized with halothane (2.5–3% in oxygen), and under aseptic conditions, the L5 and L6 spinal nerves were exposed on the left side for both sham and SNL, but tightly ligated with silk 5-0 suture only for SNL, as previously described.13

**Mechanical Hyperalgesia Assessment**

As baseline testing, before surgery and after a 13-day postoperative recovery period, animals were placed in plastic cages on a plastic mesh floor and were allowed to acclimate for 30 min. The threshold required to elicit withdrawal of the injured hind paw was tested using calibrated von Frey filaments. The tests were started using a filament that is in the middle of a series of eight von Frey filaments with logarithmically incremental stiffness (0.76, 2.65, 3.66, 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 approximately 6 s. The withdrawal thresholds were calculated using the up-down method as described previously.14 The method was modified to exclude a cutoff of 15 g. Each sham and SNL rat was tested twice at 5-min intervals, and the average of these values was used. A threshold of greater than 4 g for normal and sham rats and a threshold of 4 g or less for SNL rats was necessary for inclusion in the study.

**Synaptosome Preparation**

After induction of anesthesia with halothane, four animals were killed by decapitation for preparation of one synaptosome experiment each. The spinal cord was quickly removed, was cleaned of dura, blood vessels, and spinal roots, and was immediately placed in 0.32 M ice-cold sucrose. The cervical and lumbar dorsal halves were selected and homogenized with 15 strokes in a plastic homogenizer in 8 ml ice-cold sucrose. A crude synaptosomal pellet (P2) was prepared by differential centrifugation at 1,000g followed by 20,000g, as previously described.6 The synaptosomal P2 pellet was resuspended in 8 ml oxygenated (95% O2; 5% CO2) Krebs-Henseleit bicarbonate medium (containing 111 mM NaCl, 1.2 mM NaH2PO4, 26.2 mM NaHCO3, 4.7 mM KCl, 1.8 mM CaCl2, 1.2 mM MgCl2, and 11 mM glucose at pH 7.4) and was incubated at 37°C with periodic gassing.

**Adenosine Release**

Adenosine release was induced by morphine as described previously.6 Briefly, after centrifugation at 20,000g for 4 min at room temperature, the synaptosomal P2 pellet was resuspended into 3.6 ml Krebs-Henseleit medium. For the morphine experiments, aliquots of 450 μl of the synaptosomal P2 pellet suspension were pipetted into six test tubes with 50 μl of solution containing buffer alone or with morphine at final concentrations of 0, 10−8, 10−7, 10−6, 10−5, and 10−4 M. For the morphine and dipyridamole experiment, 400 μl of the synaptosomal P2 pellet suspension was pipetted into six test tubes with 50 μl morphine as described together with 50 μl of dipyridamole at a concentration of 10−5 M. A seventh test tube contained 400 μl of the synaptosomal P2 pellet suspension with 50 μl saline and 50 μl dipyridamole at a concentration of 10−4 M. The contents were mixed, and the test tubes were incubated at 37°C for 15 min followed by centrifugation at 15,000g for 4 min. Three hundred microliters supernatant was deproteinized by adding 150 μl ZnSO4 and 150 μl BaOH by final centrifugation at 15,000g for 4 min. Adenosine release was measured by high-pressure liquid chromatography with ultraviolet detection, expressed as picomoles per milligram of protein per 15 min. High-pressure liquid chromatography was performed using a Luna 2 (C 18) column from Phenomenex (Torrance, CA), 250 × 4 mm, with a Waters 515 pump (Waters Corp., Milford, MA) to deliver the mobile phase (10−3 M ammonium phosphate; 16% methanol; pH 6.0) at 1.3 ml/min. In each assay, 20-μl samples were injected through a Raninn AI-1A autosampler (Varian Inc., Walnut Creek, CA) and were detected at 254 nm. Values were corrected for protein content.15

**Drugs**

Drugs used and their sources were as follows: morphine sulfate (Astra Pharmaceutical Products, Inc., Westborough, MA); barium hydrochloride monohydrate (Aldrich Chemical Company, Inc., Milwaukee, WI); dipyridamole, HEPES, sucrose, sodium phosphate monobasic, and ammonium phosphate monobasic (Sigma Chemical Co., St. Louis, MO); methanol, zinc sulfate, sodium chloride, sodium bicarbonate, and glucose (Fisher Scientific, Pittsburgh, PA); calcium chloride dihydrate, potassium chloride, and magnesium chloride (Fluka Chemica AG, Buchs, Switzerland). Morphine sulfate was diluted in 0.9% physiologic saline, dipyridamole in Krebs-Henseleit medium.

**Data Analysis**

All release experiments were performed as a set of five or eight synaptosome preparations. Each synaptosome preparation contained dorsal spinal halves of four rats. Values are expressed as mean ± SEM. Adenosine release was converted to percent of control adenosine release, which was defined as: $100 \times$ postdrug response/buffer response. Groups were compared by two-way repeated measures analysis of variance, with the Dunnett test used for post hoc comparison with baseline. A P value of less than 0.05 was considered significant.

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Results

Behavioral Studies

Postoperative withdrawal threshold decreased to less than 4 g in all animals after SNL but not after sham surgery. All normal animals showed a threshold of more than 4 g. Therefore, all animals could be included in the following synaptosome study.

Synaptosomes

No differences in morphine-induced adenosine release of synaptosomes were detected between the lumbar spinal cord of sham animals and the cervical or lumbar spinal cord of normal animals (fig. 1). Incubation of synaptosomes from the cervical spinal cord with morphine resulted in a concentration-dependent release of adenosine, which was similar in normal and SNL rats (fig. 2A). In contrast, morphine-induced adenosine release was considerably less in lumbar cord synaptosomes from SNL animals than from normal animals ($P < 0.05$; fig. 2B). Dipyridamole alone led to 13% and 16% increases in adenosine release in normal and SNL rats, respectively ($P < 0.05$). Addition of $10^{-5} \text{M}$ dipyridamole to morphine increased adenosine release compared with morphine alone in SNL rats (fig. 3A) but not in normal rats (fig. 3B). This selective effect of dipyridamole in SNL rats resulted in a level of morphine-induced adenosine release that was indistinguishable from that of normal animals.

Discussion

Although others have previously demonstrated that opioids stimulate adenosine release in the spinal cord, these are the first direct observations to suggest this opioid-adenosine link is disrupted in an animal model of neuropathic pain. Accumulating evidence indicates that the antinociceptive effects of opioids are in part mediated by endogenous adenosine. Interest in the involvement of adenosine in morphine's analgesic activity on the brain was suggested by the demonstration of morphine- or opioid-evoked central adenosine release. Therefore, opioids may conceivably exert some of their actions by increasing extracellular concentrations of adenosine in the brain. Morphine also enhances adenosine release induced by depolarization with veratridine in brain slices.

The sources of adenosine release from morphine in the spinal cord are uncertain but clearly include small-diameter primary afferent nerve terminals. Pharmacologic studies indicate that morphine stimulates spinal adeno-
A

![Graph A](image)

B

![Graph B](image)

**Fig. 3.** Adenosine release (percent of control without drug exposure; mean ± SE) as a function of morphine concentration with or without additional 10⁻⁵ M dipyridamole in lumbar dorsal spinal cord synaptosomes (A) from spinal nerve ligated rats and (B) from normal rats (morphine group, n = 8 experiments/32 rats; morphine + dipyridamole group, n = 5 experiments/20 rats). Baseline (= 100%) represents the percent of control without drug exposure: lumbar-SNL-morphine, 372.59 ± 38.91 pmol·mg⁻¹ protein·15 min⁻¹; in lumbar–SNL-morphine-dipyridamole, 368.22 ± 23.68 pmol·mg⁻¹ protein·15 min⁻¹; lumbar–normal–morphine, 364.00 ± 26.25 pmol·mg⁻¹ protein·15 min⁻¹; lumbar–normal–morphine–dipyridamole, 400.9 ± 44.14 pmol·mg⁻¹ protein·15 min⁻¹.

Various methylxanthine adenosine receptor antagonists blocked the spinal neuronal depressant effects of morphine.⁵ In the periphery, the adenosine A1 receptor has been proposed to associate in a complex fashion with μ-opioid receptors.¹⁸ These findings support the concept that adenosine has a role in opioid-induced analgesia. Clinically, a dose of 0.1–0.3 mg morphine is used as an intrathecal bolus in combination with local anesthetics,¹ which should result in an initial concentration of 10⁻⁶ to 10⁻⁵ M, within the concentration range that we studied. Several explanations for reduced morphine-induced adenosine release after spinal nerve injury are possible. It could reflect changes in spinal opioid receptors, such as (1) a segmental decrease in μ-opioid receptor expression in the spinal cord, (2) a decrease in the affinity of morphine for the receptor, (3) a decrease in the fraction of μ-opioid receptors present in the high-affinity state, or (4) a reduction in the ability of morphine to activate signal transduction, e.g., G proteins, via the μ-opioid receptor. A recently published study addressing these hypotheses in SNL rats found minimal and discrete rather than generalized loss or changes of μ-opioid receptors in the spinal dorsal horn ipsilateral to nerve injury, suggesting that the loss of spinal opioid potency and efficacy seen after nerve injury may be due to factors other than receptor number or signal transduction.²¹ Opioid-evoked release of adenosine seems to differ from nucleotide-derived basal adenosine release,⁶,⁸ but the precise source of opioid-evoked adenosine is not fully understood.⁹,¹⁹ The transport of adenosine across the cell membrane is a near-equilibrium process controlled primarily by intracellular and extracellular concentrations of adenosine. A decreased release of adenosine from synaptosomes could reflect alterations in the rapidly acting facilitated-diffusion nucleoside transport mechanism of adenosine after nerve injury. It is conceivable that increased activity of the injured nerve results in less free, unbound adenosine in the cell’s cytosol, resulting in an immediate re-uptake of adenosine after morphine-induced release of adenosine.²² This would imply that morphine induces release of similar amounts of adenosine in normal and SNL animals, but the rapidly acting re-uptake reduces the measurable extracellular adenosine concentration and that available to stimulate adenosine receptors after nerve injury. Therefore, it can be assumed that inhibition of adenosine re-uptake selectively increases extrasynaptosomal adenosine in SNL more than in normal animals.

Inhibition of adenosine reuptake enhances spinal opioid antinociception at the spinal level in normal animals⁶,²⁵ and in those after spinal nerve injury.⁵ Dipyridamole, an inhibitor of the facilitated-diffusion nucleoside transporter, increases extracellular adenosine concentrations in spinal tissue from normal animals and potentiates the analgesic effect of morphine in a dose-dependent manner in both the hot-plate and tail-immersion tests.²⁴ We observed a 13–16% increase in extrasynaptosomal adenosine over baseline in normal and SNL animals, respectively, due to dipyridamole alone. After adding morphine, no difference was seen between morphine and dipyridamole-morphine–induced adenosine release in synaptosomes of normal rats. Because dipyridamole blocks the nucleoside transporter in both directions, one could presume that the increased extrasynaptosomal adenosine from dipyridamole reflects extracellular degradation of nucleotides. This is unlikely because dipyridamole itself does not affect adenosine triphosphate release,⁹ and morphine-induced extrasynaptosomal adenosine is not derived from nucleotides.⁸ In...
previous animal studies, \(10^{-5}\) m dipyridamole alone produced a complete block of the uptake of exogenous adenosine, suggesting that this dipyridamole-sensitive carrier is responsible for most, if not all, of the removal of extracellular adenosine from the synaptic cleft. These observations support the hypothesis that dipyridamole functions in synaptosomes mainly as a re-uptake inhibitor, explaining the reversal of the decrease of morphine-induced adenosine release in SNL rats by dipyridamole.

A recent study suggests that oral dipyridamole might improve opioid efficacy in patients with neuropathic pain. In an open label trial in 15 patients with chronic pain, dipyridamole improved pain relief in 47% of cases. Six of 15 patients took opioids, but no mention was made to a possible benefit of the dipyridamole–opioid combination. At therapeutic plasma concentrations in humans, intravenous dipyridamole increases plasma concentrations of adenosine. In rats, centrally applied dipyridamole increases adenosine concentrations in the cerebrospinal fluid. These observations suggest a higher efficacy of intrathecal than intravenous dipyridamole for treatment of neuropathic pain. However, it is inappropriate to administer intrathecal dipyridamole in humans because its safety has not been examined. Controlled clinical trials of systemic dipyridamole as an add-on therapy to opioids for neuropathic pain are clearly supported by the current study.

In summary, morphine releases significantly less adenosine from lumbar dorsal spinal cord synaptosomes from rats with SNL and mechanical hypersensitivity than from normal or sham rats. This reduced release was increased to normal values by the nucleoside transport inhibitor dipyridamole. These data show a dipyridamole-sensitive disruption in the opioid–adenosine link in the spinal cord as one explanation for decreased efficacy and potency of opioids in the treatment of neuropathic pain.

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