Spinal Nitric Oxide Mediates Antinociception from Intravenous Morphine

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Introduction: Spinal nitric oxide (NO) is thought in many circumstances to play a pronociceptive role, because spinal injection of NO synthase inhibitors block hypersensitivity after nerve injury and enhance antinociception from spinal opioids. Conversely, intravenous injection of morphine has been demonstrated to activate descending noradrenergic pathways and to increase spinal synthesis of NO. This study examined the role of spinal NO in antinociception produced by intravenously administered morphine.

Methods: Polyethylene catheters were inserted with tips in the lumbar intrathecal space and in a jugular vein in male rats. Antinociception in response to intravenous injection of morphine was determined by latency to withdrawal of the hind paw from a heat source. Animals received an intrathecal injection of saline, an α2-adrenergic antagonist (idazoxan), a muscarinic antagonist (atropine), two NO synthase inhibitors, or an NO scavenger after intravenously administered morphine.

Results: Intravenously administered morphine produced dose-dependent antinociception, which was stable for 45 min and unaffected by intrathecally administered saline or atropine injection. In contrast, idazoxan, each of the NO synthase inhibitors, and the NO scavenger produced dose-dependent attenuation of intravenously administered morphine-induced antinociception.

Discussion: These results confirm a spinal α2-adrenergic mechanism of antinociception from intravenously administered morphine, consistent with morphine’s activation of descending noradrenergic pathways. Further, these data suggest that spinal NO mediates antinociception produced by intravenous morphine. (Key words: Analgesia; intrathecal; norepinephrine; opioid; pain.)

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exposure to acetylcholine. Although spinal cord microdialysate concentrations of NO also increase after intravenous injection of morphine in vivo, the role of spinal NO in the analgesic actions of morphine has not been examined extensively. The current study tested the hypothesis that spinal NO mediates antinociception from intravenously administered morphine.

**Methods**

After approval by the Animal Care and Use Committee, intrathecal and jugular vein catheters were inserted in halothane-anesthetized adult male Sprague-Dawley rats as previously described. Intrathecal catheters were advanced 8.5 cm caudal through an incision in the cisternal membrane. Location of the catheter tip was confirmed in some but not all animals by post mortem dissection. At least 3 days elapsed after recovery from anesthesia before study, and only animals with no evidence of neurologic deficit after insertion of catheters were studied.

Antinociception was determined by latency to paw withdrawal from an intense light focused on the hind paw as previously described. Animals were acclimated to the testing apparatus and procedures until stable latencies were obtained, and the glass surface on which the animals rested was maintained at 30°C during all testing. Light intensity was adjusted so that baseline latency was between 10 and 15 s in all animals, and a cutoff of 30 s was not exceeded to avoid tissue damage during periods of analgesia. Because use of this cutoff resulted in censored data, all latencies were converted to percent maximum possible effect according to the standard formula: (Observed latency − Baseline latency)/(30 − Baseline latency) × 100. Each animal was studied two or three times, with experiments separated by at least 3 days.

Antinociception from intravenously administered morphine alone, its duration of action, and the effect of intrathecal injection of saline on this antinociception were determined initially. Animals received intravenous bolus injections, administered in a volume of 0.5 ml for 10-15 s at 20-min intervals to cumulative doses of 0.5, 1.5, and 4.5 mg/kg. Withdrawal latency was determined from each hind paw before end at 5-min intervals after injection of morphine. In this and all subsequent studies, withdrawal latencies in right and left paw were averaged at each time interval. After construction of this dose-response relation, a dose predicted to provide a maximum possible effect of ≈60% (2.5 mg/kg) was injected intravenously, and withdrawal latencies were determined at 5-min intervals for 2 h after injection of morphine. Based on these studies, it was determined that antinociception from intravenously administered morphine was stable for at least 45 min after injection.

Next, the spinal pharmacology of intravenously administered morphine antinociception was examined. Animals received 2.5 mg/kg morphine administered intravenously, followed in 20 min by intrathecal injection of saline or a drug, and followed in 15 min by a second intrathecal injection of saline or a greater dose of the same drug. By this method, two doses of drug were studied in each experiment. Drugs studied were the α2-adrenergic antagonist idazoxan (2.5-45.0 μg), the muscarinic antagonist atropine (5-40 μg), the nonspecific NOS inhibitor Nω-monomethyl-L-arginine (NMMA; 2-36 μg), the neuronal NOS specific inhibitor 1-(2-trifluoromethylphenyl) imidazole (TRIM; 2.5-45.0 μg), and the NO scavenger 2-(4-Carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide potassium (PTIO; 2.5-45.0 μg). To study the interaction between intravenously administered morphine and increased spinal availability of NO, other animals, using the same paradigm, received morphine administered intravenously followed by intrathecal injection of l-arginine (20-400 μg) or the NO donor S-nitroso-N-acetylpenicillamine (SNAP; 20-200 μg). In control experiments, five to seven animals received the largest dose of each of the intrathecal agents listed here in the absence of morphine. Animal activity after drug injection was noted with each experiment. There were five to seven animals in each experiment, and the investigator was not blinded to the intrathecally injected drug.

Drugs given by intrathecal injection were dissolved in normal saline and administered in a volume of 5 μl, followed by a 10-μl flush with normal saline. Atropine, l-arginine, PTIO, NMMA, morphine, idazoxan, and SNAP were obtained from Sigma Chemical Co. (St. Louis, MO). SNAP and TRIM were obtained from Calbiochem (San Diego, CA).

Data are presented as mean ± SD. Dose–response relations were determined by linear regression analysis, and effects of individual doses were determined by one-way analysis of variance followed by Dunnett’s test. A probability value <0.05 was considered statistically significant.

**Results**

In all cases examined, the tip of the intrathecal catheter resided in the lumbar intrathecal space. Withdrawal
SPINAL NO AND ANTINOICEPTION FROM MORPHINE

![Graph](attachment:image.png)

Fig. 1. Effect of intravenously administered morphine alone on paw withdrawal response to noxious heat. Morphine (2.5 mg/kg) produced an increase in paw withdrawal latency, expressed as percent maximum possible effect (%MPE), which was stable for 60 min in the presence of repeated intrathecal injections of saline (top). Arrows indicate time of saline injection. Morphine antinoiception was linearly related to the log of the dose (bottom). Data are presented as mean ± SD.

Latencies were 10–15 s in each animal before administration of the drug. Intravenously administered morphine produced dose-dependent antinoiception, with 2.3 ± 0.4 mg/kg as the effective dose in 50% of subjects (fig. 1). The effect of intravenously administered morphine (2.5 mg/kg) was maximum within 15 min, stable for 50–60 min, and unaffected by intrathecal injection of saline (fig. 1).

The α2-adrenergic antagonist idazoxan, but not the muscarinic antagonist atropine, produced a dose-dependent blockade of antinoiception from intravenously administered morphine (fig. 2). The nonspecific NOS inhibitor NMMA and the specific neuronal NOS inhibitor TRIM produced a dose-dependent attenuation of antinoiception from intravenously administered morphine, as did the NO scavenger PTIO (fig. 3). Analysis of the dose–response relation of each drug suggested a similar potency of all drugs (table 1).

Neither intrathecal injection of L-arginine nor SNAP affected antinoiception from intravenously administered morphine (fig. 4). Animals were sedated, as evidenced by inactivity, after the largest dose of SNAP, although SNAP did not alter withdrawal latency. There were no sedation effects, defined as inactivity, from the other agents studied. Withdrawal latency remained within 2 s of control values after injection of intrathecal agents without intravenously administered morphine.

**Discussion**

Activation of descending spinal inhibition represents a major mechanism of analgesia from systemically administered opioids. A variety of descending pathways are thought to be activated by opioids, which in turn may trigger a variety of spinal circuits to produce their effect. Evidence for a spinal norepinephrine → acetyl-
choline → NO mechanism of analgesia from opioids has relied primarily on measurement of neurotransmitters in cerebrospinal fluid or in spinal cord interstitial fluid by microdialysis. Therefore, these functional data in the behaving animals provide unique and complementary evidence to support the relevance of this proposed mechanism.

The results of the current study and those from previous studies do not unequivocally support the systemic opioid → spinal norepinephrine → spinal acetylcholine portion of the hypothesis described here. First, intrathecal injection of the noradrenergic (α2-adrenergic) antagonist attenuated intravenously administered morphine antinociception, which is consistent with previous work and with intravenously administered opioid-induced increases in norepinephrine in cerebrospinal fluid in humans and in microdialysates from the dorsal horn in sheep. This is consistent with the systemic opioid → spinal norepinephrine portion; however, intrathecal injection of the muscarinic antagonist atropine failed to attenuate intravenously administered morphine-induced antinociception in the current study, just as it failed to attenuate antinociception from intra-

Table 1. Potency of Intrathecal Agents to Attenuate Morphine Antinociception

<table>
<thead>
<tr>
<th>Drug</th>
<th>ID50 (μg)</th>
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<tbody>
<tr>
<td>Idazoxan</td>
<td>24 ± 11</td>
</tr>
<tr>
<td>NMMA</td>
<td>31 ± 18</td>
</tr>
<tr>
<td>TRIM</td>
<td>24 ± 14</td>
</tr>
<tr>
<td>PTIO</td>
<td>34 ± 24</td>
</tr>
</tbody>
</table>

Data are mean ± SD of regression analysis from five or six rats at each drug dose and are expressed as dose producing a 50% inhibition of the effect of morphine, 2.5 mg/kg (ID50).

NMMA = Nω-monomethyl-L-arginine; TRIM = 1-(2-fluoromethylphenyl) imidazole; PTIO = 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazole-1-oxyl 3-oxide.
spinally administered α₂-adrenergic agonists in sheep and rats. Attenuation by atropine would be expected according to the systemic opioid — spinal norepinephrine — spinal acetylcholine hypothesis.

Other experiments support the role of spinal released acetylcholine in analgesia from systemic opioids and spinal α₂-adrenergic agonists. Therefore, intrathecal injection of the α₂-adrenergic agonist clonidine increases concentrations of acetylcholine in cerebrospinal fluid in sheep and humans. In some studies, antinociception from systemic morphine and from intrathecally administered clonidine is antagonized by intrathecally administered atropine, and the literature is uniform on the potentiation of antinociception from systemic opioids and intraspinal α₂-adrenergic agonists by intrathecally administered cholinesterase inhibitors in humans, sheep, and rats. The origin of this discrepancy over the role of spinal acetylcholine in systemic opioid analgesia in several species is uncertain and not resolved by the experiments in the current study.

As described here, most evidence favors a pronociceptive action of spinal NO during many conditions. These results, however, like those with acetylcholine described earlier, are not without contradictions. Systemic administration of the NO substrate l-arginine has been shown to produce analgesia in animals and in humans with chronic pain. Administration of NO donors has been shown to inhibit spontaneously active neurons in the superficial dorsal horn of rats and to diminish evoked substance P and calcitonin gene-related peptide release in spinal cord slices in vitro, which is consistent with analgesic actions. Mice lacking neuronal NOS gene expression also develop hyperalgesia and allodynia after injection of formalin, but this is not blocked by NOS inhibitors, suggesting that other mechanisms may yield this hypersensitivity.

Although enhancement of opioid antinociception from NOS inhibitors has been demonstrated repeatedly when both agents are administered by the same route, either systemically, supraspinally, or intrathecally, these experiments do not address directly the possibility that systemic opioids may stimulate synthesis of NO in the spinal cord for analgesia. In mice, antinociception from intracerebroventricular administration of β-endorphin, but not morphine, is diminished by intrathecal injection of NOS inhibitors. We have demonstrated increased concentrations of NO in spinal cord microdialysates from intrathecally administered morphine, although the functional relevance of this NO was not known. The current study, using two NOS inhibitors, at least one of which is considered to be specific for the neuronal form of NOS and an NO scavenger, suggests that this spinal released NO contributes to analgesia from intravenously administered morphine.

Neuronal NOS has been suggested recently to exist in two forms, representing two splice variants, in the spinal cords of mice. Selective reduction of one of these splice variants by intrathecal administration of an antisense oligodeoxynucleotide inhibits the development of tolerance to morphine, whereas selective reduction of the other reduces the antinociception to a heat stimulus produced by intravenously administered morphine in mice. These data support the current pharmacologic study in rats, suggesting an antinociceptive role for spinal NO after intravenous administration of morphine.

Although spinal NOS inhibitors reduced antinociception from intravenously administered morphine in the current study, intrathecal injection of the NOS substrate l-arginine or of the direct NO donor SNAP had no potentiating effect on intravenously administered morphine antinociception, nor did they produce antinociception themselves. These data are consistent with previous reports showing minimal or no antinociception from l-arginine or NO donors after intrathecal administration. As reviewed earlier, spinal NO has been implicated in pronociceptive actions and in opioid antagonistic effects during some circumstances. It is conceivable that administration of an NO donor intrathecally may result in stimulation of pro- and antinociceptive sites of action, in contrast to the synaptically stimulated NO generation postulated from intravenous administration of morphine. Similarly, the ability of l-arginine to stimulate synthesis of NO during basal conditions has not been demonstrated uniformly.

The source of NO responsible for the attenuation of antinociception of intravenous morphine by intrathecal NOS inhibitors was not addressed in the current study. Nitric oxide synthase has been identified in glia, interneurons, and fibers in the spinal cord. In support of an analgesic role of spinal NO and its interaction with acetylcholine, cells and fibers in the dorsal horn that contain NOS also contain the classic inhibitory neurotransmitter γ-aminobutyric acid and acetylcholine.

Antinociception from intravenously administered morphine is reduced by intrathecal injection of α₂-adrenergic and NOS inhibitors. These data suggest that increases in spinal NO observed in animals and humans...
after intravenous administration of morphine likely play a functional role in mediating the analgesic action of morphine. The role of spinaly released acetylcholine in antinociception from intravenously administered morphine is not clear.

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