Intravenous Morphine Increases Release of Nitric Oxide From Spinal Cord by an α-Adrenergic and Cholinergic Mechanism

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Xu, Zemin, Chuanyao Tong, Hui-Lin Pan, Sergio E. Cerda, and James C. Eisenach. Intravenous morphine increases release of nitric oxide from spinal cord by an α-adrenergic and cholinergic mechanism. J. Neurophysiol. 78: 2072–2078, 1997. Systemic opioids produce analgesia in part by activating bulbospinal noradrenergic pathways. Spinally released norepinephrine (NE) has been suggested to produce analgesia in part by stimulating α2-adrenoceptors on cholinergic spinal interneurons to release acetylcholine (ACh). We hypothesized that this spinally released ACh would stimulate synthesis of nitric oxide (NO), and that spinally released NO after intravenous (IV) opioid injection would depend on a cascade of noradrenergic and cholinergic receptor stimulation. To test these hypotheses, IV morphine was administered to anesthetized sheep, and neurotransmitters in dorsal horn interstitial fluid were measured by microdialysis. IV morphine increased NE and ACh in dorsal horn microdialysates, and these increases were inhibited by IV naloxone or cervical spinal cord transaction. IV morphine also increased dorsal horn microdialysate concentrations of nitrite, a stable metabolite of NO. Increases in NE, ACh, and nitrite were antagonized by prior intrathecal injection of the α2-adrenergic antagonist idazoxan, the muscarinic antagonist atropine, or the NO synthase inhibitor N-methyl-L-arginine (NMLA). To examine the concentration-dependent effects of spinal adrenergic stimulation, isolated rat spinal cord tissue was perfused with the α2-adrenergic agonist clonidine. Clonidine increased nitrite in the spinal cord tissue perfusate, an effect blocked by coadministration of idazoxan, atropine, and NMLA. These data support a previously hypothesized cascade of spinally released NE and ACh after systemic opioid administration. These data also suggest that spinally released NO plays a role in the analgesic effects of systemic opioids. In addition, these data imply a positive feedback whereby spinally released nitric oxide increases NE release and that has not previously been described.

INTRODUCTION

Opioids produce analgesia by actions at several sites, but a major mechanism involves activation of bulbospinal inhibitory, primarily serotonergic (Yaksh and Tyce 1979) and noradrenergic (Tyce and Yaksh 1981) pathways. Thus systemic or intracerebral administration of opioids increases norepinephrine (NE) concentrations in lumbar cerebrospinal fluid (CSF) (Tyce and Yaksh 1981) and microdialysates from spinal cord dorsal, but not ventral horn (Bouaziz et al. 1996). This spinally released NE produces analgesia by stimulation of α2-adrenoceptors, because analgesia from spinally administered NE (Howe et al. 1983) or from centrally administered opioids (Tseng and Tang 1989) is blocked by spinal injection of α-adrenergic antagonists. In addition, augmentation of synaptic NE availability by inhibition of NE reuptake in the spinal cord potentiates analgesia from centrally administered morphine (Taiwo et al. 1985).

It recently has been suggested that spinally released NE produces analgesia in part by activating spinal cholinergic interneurons to release acetylcholine (ACh). Thus spinal injection of α2-adrenergic agonists increases ACh concentrations in CSF (Detweiler et al. 1993) and microdialysates from spinal cord dorsal but not ventral horn (Klimscha et al. 1995). Acetylcholine produces analgesia when administered spinally, an effect blocked by muscarinic receptor antagonists (Yaksh et al. 1985). Furthermore, centrally administered opioids increase ACh concentrations in CSF and spinal cord dorsal horn microdialysates (Bouaziz et al. 1996), and analgesia from central opioid injection is partially reversed by spinal injection of muscarinic receptor agonists (Dirksen and Nijhuis 1983).

Acetylcholine stimulates synthesis of nitric oxide (NO) in vascular endothelium, and it has been hypothesized that ACh similarly stimulates NO synthesis in the spinal cord (Iwamoto and Marion 1994a; Zhuo et al. 1993). In this regard, ACh has been shown to stimulate release of a vasorelaxant from spinal cord tissue in vitro that shares the pharmacology of NO (Xu et al. 1996b), and analgesia from intrathecal injection of muscarinic receptor agonists is blocked partially by NO synthase (NOS) inhibitors (Iwamoto and Marion 1994a). These results suggest that spinally released NO may play a role in analgesia produced by intravenous (IV) morphine, involving a cascade of NE and ACh.

Although the above data would suggest that systemically administered opioids may activate NOS in the spinal cord, this has not previously been investigated. In contrast, considerable evidence suggests an antagonism of opioid analgesia by NO and a pro-nociceptive role for spinal NO. Spinally released NO has been implicated in the production of hyperalgesic states after neural injury or application of a chronic noxious stimulus (Meller and Gebhart 1993). Systemically administered NOS inhibitors enhance, rather than inhibit, analgesia from systemically administered opioids (Yamaguchi and Naito 1996). Similarly, systemically administered NOS inhibitors prevent the development of tolerance from systemically administered opioids (Kolesnikov et al. 1992). Although these studies support a pronociceptive effect of spinally released NO under pathological conditions and opposing effects of opioids and NO with systemic administration, previous studies have not addressed the interaction between systemic opioids and spinal NOS activity.

The purpose of the current study was to confirm, using
in vivo microdialysis, the proposed systemic opioid-induced spinal NE and ACh cascade and to test the hypothesis that this cascade also involves the release of NO. We hypothesized that intrathecal injection of an α₂-adrenergic antagonist would block the IV morphine-induced spinal release of ACh and NO and that intrathecal injection of a cholinergic receptor antagonist or a NOS inhibitor would block the increase in spinal NO after intravenous morphine. To avoid confounding factors of agents on tissue blood flow and redistribution in vivo, more detailed concentration-response studies also were performed on rat spinal cord tissue in vitro.

**METHODS**

**Animal preparation**

Following animal care and use committee approval, microdialysis experiments were performed on a total of 27 ewes of mixed Western breeds (weight range, 40–50 kg). Sheep were chosen because of their larger spinal cord than rodents, allowing precise localization of microdialysis catheters and destruction of relative less spinal cord tissue by the catheters. The animals were fasted for 24 h, then anesthesia was induced with ketamine (5–10 mg/kg im). The trachea was intubated and anesthesia maintained with halothane, 1–2%, 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 laminectomy was performed at the lumbar sacral 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 2.5 cm cephalad so that its tip was at the midthoracic 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. During this time the animals received daily penicillin G (900,000 units im).

**Surgical procedure**

On the day of the experiment, after a 24-h fast, anesthesia was induced with thiopentone sodium (5 mg/kg IV) and maintained with halothane, 1–2%, during the surgical preparation. Subsequently during the microdialysis experiment, anesthesia was maintained with halothane, 0.5%, in oxygen by controlled ventilation and muscle relaxation with pancuronium (0.1 mg/kg IV, every 2 h). Ventilation was adjusted to keep PaCO₂ within a normal range by continuous monitoring of end tidal CO₂ and intermittent monitoring of arterial blood gas tensions. Blood pressure and heart rate were continuously monitored via the femoral artery catheter and recorded throughout the experiment.

The animal was turned prone and a five-level, bilateral laminectomy was performed in the midthoracic region, leaving the dura and portions of the dorsal spinal processes intact for stability. Six to 10 microdialysis probes were inserted transversely through the superficial dorsal spinal cord at different sites along the exposed cord. After completion of the experiment, location of the area of praspinal sites, a spinal cord perfusion method was developed. To examine the interactions at the spinal level of noradrenergic, cholinergic, and nitric activation without the confounding factors of effects of compounds on blood flow or distribution to supraspinal sites, a spinal cord perfusion method was used. Adult male Sprague-Dawley rats were euthanized with sodium pentobarbital (50 mg/kg ip) and the spinal cord removed. Each spinal cord was divided into two parts, then chopped in 0.5-mm slices. Tissue sections from each hemispinal cord were put into an incubation chamber surrounded by a temperature-controlled water bath maintained at 37°C. Tissue slices were perfused continuously with a multi-channel pump at 0.5 ml/min with oxygenated modified Krebs-Henseleit solution [composition (in mM)] was 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 0.027 ethylenediamine tetraacetic acid (EDTA), 5 N-2-hydroxyethylpi-

**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 defined precisely using two pieces of silica tubing (SGE, Ringwood, Australia), which 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 microdialysis catheter was inserted through the dura mater and cord with minimal tissue damage. After insertion, the portion of the silica tubing connected to the wire was cut and removed to allow perfusion. The inlet of the probe was perfused continuously via a pump at a rate of 2 μl/min with artificial CSF at the following composition (in mM): 145 NaCl, 2.7 KCl, 1.0 MgCl₂, 1.2 CaCl₂, and 2.0 Na₂HPO₄ in filtered deionized water. The microdialysate effluent was collected every 15 min into cooled minitubes. The first 90 min of perfusion were considered as the washout period, allowing for tissue recovery and were followed by a 60-min collection (4 samples) for baseline. This was followed by intrathecal injection of saline (6 animals), 1 mg of idazoxan (4 animals), 1 mg of atropine (3 animals), 2 mg of yohimbine (3 animals), or 3 mg of NMLA (4 animals). All intrathecal injections were made in a volume of 1 ml followed by a 0.5 ml saline flush. Fifteen minutes later morphine (1 mg/kg) was injected intravenously. Samples then were collected every 15 min for 2 h after IV drug administration. To determine an action of morphine at supraspinal sites activating descending pathways, a complete transection of the cervical spinal cord was performed in four sheep at the end of the microdialysis probe insertion and 150 min before IV morphine administration. To confirm an action of morphine on opioid receptors, two sheep received naloxone (1 mg/kg IV) 15 min before morphine injection. Drug experiments were performed in a single blind manner.

**Neurochemical assays**

After collection, samples were fast frozen to −20°C and stored at −70°C until assay. NE concentrations were determined by high pressure liquid chromatography (HPLC) with electrochemical detection. This method has an interassay coefficient of variation of <9% for NE and an absolute detection limit of 12 fmol (Eisenach et al. 1992). ACh concentrations were determined by a different HPLC-electrochemical detection method, using other equipment than that for catecholamines. This method has an interassay coefficient of variation of 8% and a detection limit of 50 fmol (Dettweiler et al. 1993). Nitrite, a stable metabolite of NO (Salter et al. 1996), was measured via a variant of the Greiss reaction with the production of a fluorescent adduct (Miles et al. 1996), with an absolute detection limit of 100 pmol.

**Spinal cord tissue perfusion**

To examine the interactions at the spinal level of noradrenergic, cholinergic, and nitric activation without the confounding factors of effects of compounds on blood flow or distribution to supraspinal sites, a spinal cord perfusion method was developed. Adult male Sprague-Dawley rats were euthanized with sodium pentobarbital (50 mg/kg ip) and the spinal cord removed. Each spinal cord was divided into two parts, then chopped in 0.5-mm slices. Tissue sections from each hemispinal cord were put into an incubation chamber surrounded by a temperature-controlled water bath maintained at 37°C. Tissue slices were perfused continuously with a multi-channel pump at 0.5 ml/min with oxygenated modified Krebs-Henseleit solution [composition (in mM)] was 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 0.027 ethylenediamine tetraacetic acid (EDTA), 5 N-2-hydroxyethylpi-
perazine-N'2-ethanesulfonic acid, and 11 glucose] gassed with 95% O₂-5% CO₂ at 37°C. The effluent from the spinal cord tissue chambers was collected on ice in 4-min aliquots. Experiments were started after spinal cord slices had incubated in the chamber for 60 min.

To determine concentration-dependent responses, spinal cord tissue chambers were assigned randomly to receive two escalating concentrations of clonidine, separated by 10 min. Clonidine concentration pairs were (in M) 10⁻¹³ and 10⁻¹⁰, 10⁻¹² and 10⁻⁹, 10⁻¹¹ and 10⁻⁸, 10⁻¹⁰ and 10⁻⁷, 10⁻⁹ and 10⁻⁶, and 10⁻⁸ and 10⁻⁵. At the end of the experiment, to confirm the ability of the tissue to synthesize NO, all chambers were perfused with solution containing N-methyl-D-aspartate (NMDA), 10⁻⁵ M, a known stimulator of NOS in spinal cord (Li et al. 1994). For experiments with antagonists or inhibitors (idazoxan, atropine, NMLA), chambers were perfused with 10⁻⁵ M of clonidine and with randomized, escalating concentration pairs of antagonists in the same concentration pairs as described above.

Drugs

Drugs for intrathecal injection were dissolved in sterile 0.9% saline. Atropine, clonidine, idazoxan, naloxone, NMDA, NMLA, and yohimbine were purchased from Sigma Chemical (St. Louis, MO). Halothane, ketamine, pancuronium bromide, penicillin G, thiopentone sodium, and methylene blue, were obtained from Barber Veterinary Supply, Richmond, VA.

Data analysis

Data are presented as means ± SE if normally distributed and as median ± 25th and 75th percentiles if not normally distributed. Within groups values of nitrite, ACh or NE were compared with baseline by one-way Kruskal-Wallis analysis of variance (ANOVA) on ranks followed by Dunnett’s test. The groups were compared with these variables by two-way nonparametric ANOVA followed by Newman-Keuls test. P < 0.05 was considered statistically significant.

RESULTS

Microdialysis studies

All animals recovered normally from intrathecal catheter insertion, and none exhibited behavioral deficits on the day of the experiment. Arterial blood gas tensions and pH and arterial blood pressure remained stable and within normal limits for sheep throughout the experiment in all animals.

As in previous studies (Bouaziz et al. 1996), data from consecutive pairs of microdialysis samples, representing 30 min of collection, were averaged to reduce variability. Groups did not differ in baseline concentrations of NE and ACh, but did in nitrite (Table 1). As such, data are presented as raw values for NE and ACh but as change from baseline for nitrite.

EFFECT OF IV MORPHINE ALONE. IV morphine increased NE, ACh, and nitrite in dorsal horn microdialysates (Figs. 1–3). In each case, the time course was similar with a statistically significant increase in neurotransmitter at the first sample (30 min) after IV morphine injection and continued increase for 2 h after morphine injection. Microdialysate concentrations of NE, ACh, and nitrite after cervical cord transection were similar to those in sheep with intact spinal cord (median value in intact vs. spinal cord-containing chambers was collected on ice in 4-min aliquots. Experiments were started after spinal cord slices had incubated in the chamber for 60 min.

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![FIG. 1. Effect of intravenous (IV) morphine on norepinephrine in spinal cord. Spinal cord dorsal horn microdialysate concentrations of norepinephrine after IV injection at time 0 of morphine (1 mg/kg) in halothane-anesthetized sheep. Data are from experiments in which an intrathecal injection of saline (), idazoxan (1 mg; □), N-methyl-L-arginine (NMLA; 1 mg; ●), atropine (1 mg; ◊), or yohimbine (2 mg; ▽) preceded IV morphine injection by 15 min. Each symbol represents the median of 6–10 probes. Seventy-fifth percentiles for the saline group are shown as error bars. *P < 0.05 vs. time 0.](http://jn.physiology.org/DownloadedFrom/10.2302/33.4)a.png)
**EFFECT OF **AAAAADRENERGIC ANTAGONISTS. Idazoxan, in a dose shown to antagonize the hemodynamic effects of clonidine in unanesthetized sheep (Eisenach and Tong 1991), blocked morphine-induced increases in ACh, nitrite, and NE (Figs. 1–3). To confirm that this was an action on α2-adrenoceptors, three animals received intrathecal injection of yohimbine, which antagonizes α2-adrenergic but not imidazoline receptors. Yohimbine also blocked the increase in NE after IV morphine (Fig. 1).

**DISCUSSION**

The two major findings of the current study are the increase in spinal cord microdialysate concentrations of nitrite after IV morphine administration and the blockade by idazoxan, atropine, and NMLA of increased NE in these microdialysates. Each of these observations provides novel information on the mechanism of action of systemically administered opioids.

IV or central administration of opioids has been demonstrated previously to increase NE and ACh in CSF and dorsal horn microdialysates of animals and humans (Bouaziz et al. 1993). The increase in nitrite found after clonidine was shown in control experiments to be stable during the course of the experiment.
The current study establishes their relationship. Lack of increase after cervical cord transection confirms the observation that all noradrenergic innervation of the spinal cord originates from neurons with cell bodies rostral to the cord (Dahlstrom and Fuxe 1965). Functionally, spinally released NE and ACh must participate in analgesia after systemic or central administration of opioids, because opioid analgesia is antagonized by spinal injection of noradrenergic (Yaksh 1979) and cholinergic (Dirksen and Nijhuis 1983) antagonists and is potentiated by spinal injection of NE reuptake inhibitors (Taiwo et al. 1985) and cholinesterase (Eisenach and Gebhart 1995) inhibitors in animals. In humans, NE reuptake inhibitors have not been injected spinally, but spinal injection of neostigmine has been shown to potentiate IV opioid analgesia (Hood et al. 1995).

Neuronal NOS is concentrated in the rat, sheep, and human spinal cord in neurons residing in the superficial dorsal horn, surrounding the central canal, and in the intermediolateral cell column (Terenghi et al. 1993; Xu et al. 1996a), suggesting actions of NO on sensory transduction and homeodynamic control. We and others have hypothesized that actions of ACh in these areas of the cord are mediated in part by NO synthesis (Iwamoto and Marion 1994a; Xu et al. 1996a; Zhuo et al. 1993a). As regards hemodynamic actions, spinal injection of muscarinic agonists increases preganglionic sympathetic nerve activity, blood pressure, and heart rate (Bhargava et al. 1982; Calaresu et al. 1990), as does intrathecal injection of NO donors (Lee et al. 1996). There is also considerable evidence suggesting the analgesic actions of ACh is mediated in part by NO synthesis (see below).

This is the first study to examine the effect of systemically administered opioids on the spinal synthesis of NO. IV morphine produced a clear increase in nitrite concentration, thought under conditions of microdialysis experiments to accurately reflect NO synthesis (Salter et al. 1996). Blockade of nitrite increase from IV morphine by spinal injection of atropine is also consistent with a cholinergic link in spinal NO synthesis.

We propose that IV morphine stimulates NO synthesis in the spinal cord by an NE → ACh → NO cascade, and that NE acts on α2-adrenoceptors. The action of α2-adrenergic agonists on spinal cord nitrite was tested directly in the in vitro spinal cord perfusion experiments. Clonidine increased nitrite in this system, an action antagonized by α2-adrenergic, cholinergic, and NOS antagonists or inhibitors, consistent with this hypothesis.

Previous examinations of spinal NO have focused on pro-nociceptive actions, although there is also evidence for anti-nociceptive actions. For example, antinociception from supraspinal (Iwamoto and Marion 1994b) or intrathecal (Iwamoto and Marion 1994a) administration of cholinergic agonists in rats is antagonized by NOS inhibitors. Some data suggest a tonically active spinal cholinergic antinociception in rats that is dependent on local NO production in rats (Zhuo et al. 1993). Finally, enhanced antinociception by intrathecally administered neostigmine of clonidine is blocked by NOS inhibitors in sheep (Xu et al. 1996a).

Tolerance to analgesia develops rapidly in rodents exposed to opioids, and this tolerance can be prevented by NMDA antagonists (Trujillo and Akil 1991) and NOS inhibitors (Kolesnikov et al. 1992). It has been hypothesized that activation of protein kinase C by opioids results in increased intracellular calcium availability and increased NO production (Mao et al. 1995). The current study provides the first direct evidence that opioid administration can increase spinal NO production.

Although blockade of morphine-induced microdialysate increases in ACh and nitrite by spinal injection of the α2-adrenergic antagonist, idazoxan is consistent with a spinal NE → ACh → NO cascade, its blockade of NE increase is more difficult to explain. Classically, presynaptic α2-adrenoceptors inhibit the release of NE, such that NE spillover is increased by α2-adrenergic antagonists and diminished by agonists (Langer and Hicks 1984). The current results confirm a previous report in sheep that spinal injection of idazoxan diminishes, rather than enhances, morphine-stimulated NE release (Bouaziz et al. 1996). It is possible that idazoxan could produce this effect by its interaction with nonadrenergic, imidazole receptors. However, this is unlikely because yohimbine, which does not block imidazole receptors, also diminished morphine-induced increases in spinal NE.

Other experiments have suggested a nontraditional interaction between α2-adrenoceptor stimulation and NE spillover in the spinal cord. Noxious electrical stimulation in anesthetized sheep activates descending noradrenergic pathways, as evidenced by increases in lumbar CSF NE concentrations in intact, but not in spinally transected animals (Eisenach et al. 1996). As in the current study with morphine, spinal injection of idazoxan blocked the increase in CSF NE from noxious stimulation.

If α2-adrenergic antagonists decrease spinal NE release, one would expect that α2-adrenergic agonists might increase NE release. We did not examine the effects of α2-adrenergic agonists in the current study but previously found that spinal injection of clonidine increases NE in dorsal horn microdialysis.
IV Morphine

![Diagram](image)

**FIG. 6.** Proposed cascade consistent with the current experiments. IV morphine injection stimulates descending noradrenergic pathways that release norepinephrine (NE) in the spinal cord. This NE stimulates spinal cholinergic neurons to release acetylcholine (ACh), which in turn stimulates nitric oxide (NO). Because blockade of noradrenergic or cholinergic receptors and of nitric oxide synthesis diminished the increase in NE from IV morphine, we propose a positive feedback of NO on NE release.

ysates of sheep (Klimscha et al. 1995). This is likely a direct action within the spinal cord because it occurs after local spinal injection of a small dose and occurs similarly in intact and in spinally transected animals.

Other experiments in the current study suggest this blockade of stimulated spinal NE release by idazoxan involves blockade of NO synthesis at the end of a spinal cascade. Blockade of muscarinic receptors with atropine and NOS with NMLA each blocked morphine-induced spinal NE release. These data are consistent with a feedback from the end product of the α2-adrenergic agonist → ACh → NO cascade to increase spillover of NE into interstitial fluid and CSF (Fig. 6).

The nature of this positive feedback from spinal NO to NE remains unknown. NO has been shown to facilitate evoked NE release in rat hippocampal slices (Lauth et al. 1995; Lonart and Johnson 1995) and may inhibit NE reuptake mechanisms (Kaye et al. 1997). Because spinally released NO could produce vascular relaxation, it could increase local blood flow. However, one would expect this increased blood flow to reduce NE in interstitial fluid. In contrast, we observed a reduction in NE when NOS was inhibited. NO can react with a variety of molecules in addition to its major known target of guanylate cyclase. We currently are screening several candidate compounds that may react with NO and alter NE release, reuptake, or metabolism. We speculate that these compounds play a major role in the increase in interstitial concentrations of NE observed under the conditions of microdialysis and that local blockade of their production by antagonism of α2-adrenoceptors or cholinergic receptors or by NOS inhibitors is responsible for the lack of apparent increase in such NE concentrations after IV morphine.

In summary, IV morphine administration increases NE, ACh, and nitrite in microdialysates from spinal cord dorsal horn in anesthetized sheep. Antagonist experiments and in vitro perfusion of rat spinal cord studies are consistent with activation by morphine of descending noradrenergic pathways and α2-adrenoceptor activation, leading to ACh and subsequently NO synthesis and release. These results support functional studies demonstrating spinal noradrenergic and cholinergic mechanisms of analgesia from systemically administered opioids and suggest NO may regulate NE release, reuptake, or metabolism.

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