

Anesthesiology  
1996; 85:1367-77  
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## Nitric Oxide Synthase Inhibition Modulates the Ventilatory Depressant and Antinociceptive Actions of Fourth Ventricular Infusions of Morphine in the Awake Dog

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**Background:** The role of nitric oxide (NO) production, at the brain-stem level, in ventilatory control and pain perception is poorly understood. Furthermore, it is not clear whether NO synthase (NOS) inhibition can affect morphine-induced ventilatory depression or analgesia. The central hypothesis of this investigation was that NO, at supraspinal sites, can influence ventilation and nociception and can modulate the ventilatory depressant and antinociceptive actions of morphine. Using drug delivery *via* the fourth cerebral ventricle, the authors examined the ventilatory and nociceptive effects of an NOS inhibitor and an NO donor in the presence or absence of morphine sulfate (MS).

**Methods:** The studies were performed in awake dogs that were restrained in a stanchion using a fourth ventricle to cisterna magna perfusion system. The dogs were chronically prepared with fourth ventricle and cisterna magna guide cannulae, femoral arterial/venous catheters, and a tracheostomy. Agents were prepared in a temperature- and pH-controlled artificial cerebrospinal fluid, perfused at 1 ml/min through the fourth ventricle cannula, and permitted to flow out through the cisterna magna cannula. The authors measured  $P_{aCO_2}$ , ventilatory drive (inspiratory occlusion pressures during carbon dioxide rebreathing), and nociception (hindpaw withdrawal threshold to increasing electrical current). Study groups were organized according to the following perfusion sequences (40 min each step): (1) MS (1  $\mu$ g/ml)  $\rightarrow$  MS + the NOS inhibitor, nitro-L-arginine (L-NA;  $10^{-6}$ , then  $10^{-5}$  M)  $\rightarrow$  MS + L-NA ( $10^{-5}$  M) + the NO donor, S-nitroso-acetylpenicillamine (SNAP;  $10^{-4}$  M); (2) SNAP ( $10^{-5}$  M)  $\rightarrow$  SNAP ( $10^{-4}$  M); (3) L-NA ( $10^{-6}$ , then  $10^{-5}$  M)  $\rightarrow$  L-NA ( $10^{-5}$  M) + MS (1  $\mu$ g/ml)  $\rightarrow$  L-NA ( $10^{-5}$  M) + MS + SNAP ( $10^{-4}$  M); (4) MS (1  $\mu$ g/ml)  $\rightarrow$  MS + SNAP ( $10^{-4}$  M); and (5) continuous MS (1  $\mu$ g/ml) perfusion (time control). Each perfusion sequence was preceded by a 45- to

60-min perfusion with drug-free artificial cerebrospinal fluid, during which time baseline values for each measured variable were obtained.

**Results:** Nitro-L-arginine alone dose dependently and significantly reduced  $P_{aCO_2}$  and increased the nociceptive threshold. S-nitroso-acetylpenicillamine alone did not change the ventilation or nociceptive threshold. Morphine sulfate elicited a marked increase in  $P_{aCO_2}$ , a decrease in ventilatory drive, and an increase in nociceptive threshold ( $P < 0.05$  compared with baseline). With L-NA pretreatment (sequence 3), but not posttreatment (sequence 1), MS-induced ventilatory depression, relative to baseline, was significantly attenuated. For both the L-NA pre- and posttreatment protocols, combined MS/L-NA perfusions produced a significantly greater antinociceptive effect than seen when MS was given alone. The L-NA effects on MS-induced ventilatory depression and antinociception were reversed with SNAP coadministration.

**Conclusions:** Endogenous NO, produced at supraspinal sites, acts as a ventilatory depressant and as a nociceptive mediator. When NOS is inhibited, the ventilatory depressant actions of morphine can be reduced and the antinociceptive actions of morphine can be potentiated. However, NOS inhibitor treatment is more effective in suppressing morphine-induced ventilatory depression when given before, rather than after, morphine administration. The specific mechanisms involved in these actions remain to be identified. (Key words: Analgesia; morphine. Brain: cisterna magna; fourth ventricle. Nitric oxide; nitro-L-arginine: S-nitroso-acetylpenicillamine. Ventilation: carbon dioxide response.)

IT has long been known that morphine and other opioids elicit pain relief by acting on neurons within the spinal cord and the brain. Morphine or morphinelike opioids, after systemic or intracerebroventricular administration, elicit marked ventilatory depression in addition to the intended antinociception.<sup>1,2</sup> Because ventilatory depression can be life threatening, clinicians and researchers have long tried to minimize the ventilatory depressant actions of morphine while maintaining its analgesic potency.

Evidence is accumulating that indicates a vital endogenous regulatory role for nitric oxide (NO), a known

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Received from the Department of Anesthesiology, University of Illinois at Chicago, College of Medicine, Chicago, Illinois. Submitted for publication September 16, 1995. Accepted for publication July 18, 1996.

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potent vasodilator<sup>3</sup> and neuromodulator/neurotransmitter,<sup>4</sup> in the central nervous system. Nitric oxide also has been shown to play a role in nociception<sup>5-9</sup> and respiratory function.<sup>10,11</sup> Furthermore, NO may interact with morphine and other opioids, either potentiating or negating opioid actions.<sup>9,12,13</sup> Intraspinal<sup>14-16</sup> or intravenous<sup>17</sup> administration of NO synthase (NOS) inhibitors appears to be antinociceptive, particularly in hyperalgesia models, and potentiates morphine-induced analgesia.<sup>9,13</sup> At supraspinal sites, the role of NO is controversial. Despite the fact that NO production at the brain-stem level can influence ventilation,<sup>11</sup> the effects of NO and NOS inhibitors on morphine-induced ventilatory depression are unknown.

We hypothesized that supraspinal manipulations of nitric oxide concentrations would permit us to achieve greater levels of morphine-induced analgesia without enhancing ventilatory depression. To that end, these studies were designed to evaluate whether (1) NO influences normal ventilation and nociception; (2) NO affects the ventilatory depressant actions of morphine (*i.e.*, opioid-NO interactions related to breathing); and (3) NO influences the analgesic actions of morphine (*i.e.*, opioid-NO interactions related to nociception). The experiments were performed in unanesthetized dogs using a procedure that permits assessment of the ventilatory depressant and analgesic actions of agents perfused directly into the cerebral fourth ventricle.<sup>1</sup>

## Materials and Methods

The study protocol was approved by the Institutional Animal Care and Use Committee. The treatment and handling of the dogs was in accordance with the guidelines and principles set forth in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All studies were performed on male mongrel dogs ( $n = 9$ ) weighing 25 to 30 kg. Only dogs that were free of infections and parasites, displayed a quiet temperament, and readily tolerated handling and prolonged restraint were selected for study. The dogs were prepared, according to a procedure described in an earlier report from our laboratory,<sup>1</sup> with guide cannulae (which permit insertion of spinal needles into the fourth ventricle and cisterna magna), femoral arterial-venous catheters, and a tracheostomy.

On the day of the study, the dogs were placed in a stanchion that immobilized the head and provides support for the torso. A tracheostomy tube was inserted,

and spinal needles were placed into the fourth ventricle and cisterna magna using guide cannulae fastened to the occipital skull. The femoral arterial catheter implanted for long-term use was connected to a pressure transducer for continuous monitoring of arterial pressure and heart rate. The endotracheal tube was connected to a one-way valve that permitted separation of inspired and expired air. A low oxygen flow was added to the inspired air to ensure that ventilation throughout the study would not be affected by a decline in  $P_{aO_2}$ . The  $P_{CO_2}$  of expired air was continuously monitored using a Gould capnograph (Gould Godart, BV, Bithoven Netherlands). A control artificial cerebrospinal fluid (aCSF) perfusion was initiated and the animal was permitted to stabilize for 45 to 60 min. The aCSF was maintained at 38°C,  $P_{CO_2}$  at ~45 mmHg,  $P_{O_2}$  at ~60 mmHg, and pH at ~7.32.<sup>1</sup> The infusion rate was 1 ml/min and fourth ventricular inflow pressure was maintained within 2 to 3 mmHg of the value recorded under baseline conditions (5 to 15 mmHg) through adjustments to the outflow cannula height. In the last 15 to 20 min of the control period, three to four measurements of arterial  $P_{CO_2}$ ,  $P_{O_2}$ , and pH were made; two assessments of nociceptive threshold were performed; and one measure of ventilatory drive was obtained. Nociceptive thresholds were determined by monitoring paw withdrawal during electrocutaneous stimulation. That technique uses a pair of stimulating needle electrodes, at 1 cm separation, inserted subdermally into a hindpaw. The electrode tips were fashioned from 26-gauge needles and were readily tolerated by the dogs during and after insertion. The current was applied at five stimuli per second (0.5 mv per stimulus), increased at 0.1 mA/s, from an initial current of 1 mA. The nociceptive threshold was taken as the milliamperage value at which the dog raised its paw. No other overt indications of animal discomfort (*e.g.*, vocalizations; increased ventilation) were observed during the procedure. Although this procedure has not been used before in dogs, the reliability of electrocutaneous stimulation as a method to assess analgesia or nociceptive thresholds in other species, including humans, is well documented (see Discussion).

The well-established analysis of ventilatory drive used in this study was described in detail in an earlier publication from our laboratory.<sup>1</sup> The technique assesses the amount of inspiratory effort the dog is willing to exert when challenged by the ventilatory stimulant, carbon dioxide. Briefly, the procedure involved measuring in-

Table 1. Ex

Experimental

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MS = morphine  
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Table 1. Experimental Design

Experimental Group	Objectives	Sequence	n
a	i To determine the ventilatory and nociceptive effects of MS alone	i MS (1 $\mu$ g/ml)	5
	ii To examine whether L-NA can dose-dependently affect the above actions of MS	ii MS (1 $\mu$ g/ml) + L-NA ( $10^{-6}$ M) then MS (1 $\mu$ g/ml) + L-NA ( $10^{-5}$ M)	
	iii To determine whether the L-NA effects are due to reduced NO production (i.e., can exogenous NO reverse the L-NA effects)	iii MS (1 $\mu$ g/ml) + L-NA ( $10^{-5}$ M) + SNAP ( $10^{-4}$ M) SNAP ( $10^{-5}$ M)	
b	To establish the dose-dependent ventilatory and nociceptive actions of exogenous NO alone	then SNAP ( $10^{-4}$ M)	7
c	i To establish the dose-dependent ventilatory and nociceptive effects of L-NA alone	i L-NA ( $10^{-6}$ M) then L-NA ( $10^{-5}$ M)	8
	ii* To examine whether L-NA pretreatment modifies subsequent effects of MS	ii L-NA ( $10^{-5}$ M) + MS (1 $\mu$ g/ml)	
	iii To determine whether the L-NA effects are due to reduced NO production (i.e., can exogenous NO reverse the L-NA effects)	iii L-NA ( $10^{-5}$ M) + MS (1 $\mu$ g/ml) + SNAP ( $10^{-4}$ M)	
d	To examine the ventilatory and nociceptive actions of exogenous NO given in the presence of MS	MS (1 $\mu$ g/ml) then MS (1 $\mu$ g/ml) + SNAP ( $10^{-4}$ M)	5
e	MS time-control	MS (1 $\mu$ g/ml), 3-h perfusion	4

MS = morphine sulfate; L-NA = nitro-L-arginine; SNAP = S-nitroso acetylpenicillamine. All dogs within a given experimental group were exposed to the identical sequence. Each sequence step was maintained for 40 min (group e excluded).

\* Results analyzed with respect to ventilatory and nociceptive effects of MS alone. Those data were taken from groups a, d, and e (n = 9, see Results section).

spiratory occlusion pressures at increasing levels of arterial  $P_{CO_2}$  produced during 5 min of carbon dioxide re-breathing. Thus the inspiratory limb of the circuit was occluded briefly at 30-s intervals. The negative pressure change during the initial 0.2-s after occlusion was extrapolated to 1 s and paired with the concomitantly measured  $Pa_{CO_2}$ . To facilitate comparisons, the inspiratory occlusion pressures at  $Pa_{CO_2} = 60$  mmHg ( $dp/dt_{60}$ ), as established by linear regression analysis, were used.

All agents were prepared in the aCSF solution. Our original intention was to include each dog in all of the experimental groups listed below. However, this was not always possible (loss of intraarterial catheter integrity and damage to the guide cannulae represented the major reasons for animal disqualification). Thus the number of observations included in each of the experimental manipulations described below are unequal. A minimum interval of 2 weeks was allowed between experiments to prevent the onset of morphine tolerance. There is good evidence that tolerance indeed did

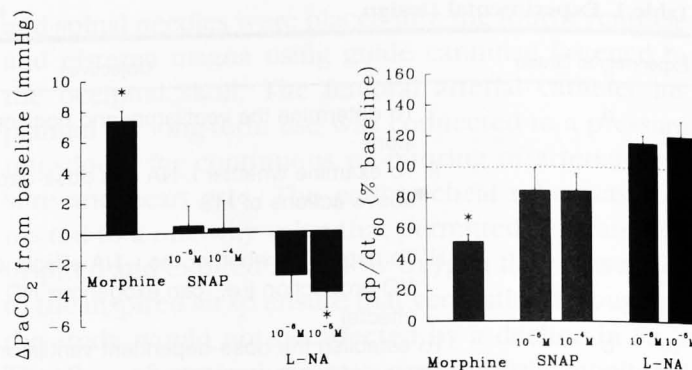
not develop in these animals. Thus, in five of the dogs, experiments with morphine as the initial agent given were performed on three separate occasions in each animal. In all five cases, subsequent treatments were not accompanied by any trends toward reductions in the antinociceptive or ventilatory depressant effects of morphine (specific data not shown). For the present experiments, we used perfusions of aCSF solutions containing morphine, increasing concentrations of inhibitors of NO synthesis, and/or NO donors. Each perfusion step was maintained for 40 min. During that time,  $Pa_{CO_2}$ ,  $Pa_{O_2}$ , and pHa were measured at 5-min intervals; nociceptive thresholds were assessed at 25 and 35 min and averaged; and ventilatory drive (carbon dioxide response) was analyzed at 40 min. Dose-response effects of morphine, with respect to ventilation, have already been established.<sup>1</sup> Those results and additional preliminary findings have indicated that a dose of 1  $\mu$ g/ml morphine sulfate is adequate to produce significant ventilatory depression and significant, but submaximal, ele-

variations in nociceptive threshold. After the initial 45- to 60-min control aCSF perfusion, five different experimental sequences were applied and designated as groups a to e. Table 1 summarizes the experimental design, including the objectives and specific sequences and doses of the drugs given for each of the five experimental groups. It should be noted that, with respect to groups a and c, exogenous NO administration was used to counter the NOS-specific actions of nitro-L-arginine (L-NA), instead of the more commonly used NO precursor and competitive NOS substrate, L-arginine. Intracerebroventricular administration of L-arginine can elicit NO-independent analgesia by promoting the release of endogenous met-enkephalin.<sup>7</sup> This clearly could complicate data interpretation. The doses of L-NA and SNAP administered were derived from pilot studies. That is, aCSF levels of L-NA  $\leq 10^{-7}$  M produced little or no change in nociceptive thresholds. Although lesser doses of SNAP induced some reversal of L-NA effects on nociception and ventilation (when present), a nearly tenfold excess of SNAP over L-NA appeared to elicit a maximal effect (*i.e.*, a complete or nearly complete reversal).

The L-NA was obtained from Sigma Chemical Company (St. Louis, MO), SNAP was obtained from Research Biochemicals Incorporated (Natick, MA), and the morphine sulfate was obtained from Eli Lilly and Company (Indianapolis, IN). Arterial  $P_{O_2}$ ,  $P_{CO_2}$ , and pH were measured in an IL BGE blood gas/pH analyzer (Instrumentation Laboratories, Lexington, MA). For statistical comparisons of the raw data within groups, we used a two-way analysis of variance, with a *post hoc* C matrix test for multiple comparisons (Systat, Evanston, IL). Comparisons of results between groups were based on data expressed relative to baseline values. That baseline data was obtained in each experiment during the initial perfusion period with drug-free aCSF. For statistical comparisons, either a multivariate analysis (Systat; for comparisons between groups a and e), or a nonparametric Kruskal-Wallis one-way analysis of variance (for comparisons of group c data with results obtained in the presence of morphine alone [data from groups a, d, and e]) was used. Differences were considered significant at  $P < 0.05$ .

## Results

Values for  $P_{aO_2}$  remained greater than 200 mmHg throughout all of the experiments and showed only minor variations. S-nitroso-acetylpenicillamine and L-NA perfusions alone were not accompanied by any signifi-



**Fig. 1.** Ventilatory changes accompanying fourth ventricular infusions of morphine (1  $\mu$ g/ml;  $n = 9$ ); the nitric oxide (NO) donor, S-nitroso-acetylpenicillamine (SNAP;  $10^{-5}$  and  $10^{-4}$  M;  $n = 7$ ); or the nitric oxide synthase (NOS) inhibitor, nitro-L-arginine (L-NA;  $10^{-6}$  and  $10^{-5}$  M;  $n = 8$ ). The measured variables were  $P_{aCO_2}$  (left) and ventilatory drive ( $dp/dt_{60}$ ; right). The values are expressed as either change from baseline ( $\Delta Pa_{CO_2}$ ) or % baseline ( $dp/dt_{60}$ ) and are presented as means  $\pm$  SE. \* $P < 0.05$  versus baseline (see table 2).

cant changes in mean arterial blood pressure. On the other hand, modest but statistically significant increases in mean arterial blood pressure were often observed after morphine perfusions were initiated. However, the maximum change observed averaged only  $18 \pm 5\%$  over baseline. Arterial pH remained near control levels except when increases in  $P_{aCO_2}$  occurred (see figs. 1 to 5). Predictably, under those circumstances, significant reductions in pH were often seen.

### Separate Actions of Morphine, SNAP, and L-NA

The results are expressed as percentage ( $dp/dt_{60}$  or nociceptive threshold) or absolute change ( $\Delta Pa_{CO_2}$ ) from baseline levels (table 2). The morphine results depicted in figures 1 and 2 were taken from each of the nine dogs used in the study. Those data were obtained in experiments in which morphine was the initial agent administered, regardless of subsequent experimental manipulations. All the morphine results shown were obtained during the same periods (*i.e.*,  $\leq 45$  min [ $n = 9$ ]) and include data from groups a, d, and e. Only one morphine-related value was permitted per dog. That is, in five of the nine dogs studied, on three separate occasions, morphine was the initial experimental drug given. In those dogs, the average of three values for  $P_{aCO_2}$ ,  $dp/dt_{60}$ , and nociceptive threshold were used (includes calculations of  $\Delta Pa_{CO_2}$ , percentage baseline  $dp/dt_{60}$ , and percentage baseline nociceptive threshold). In the remaining four dogs, only one such morphine perfusion was performed. Morphine, as ex-



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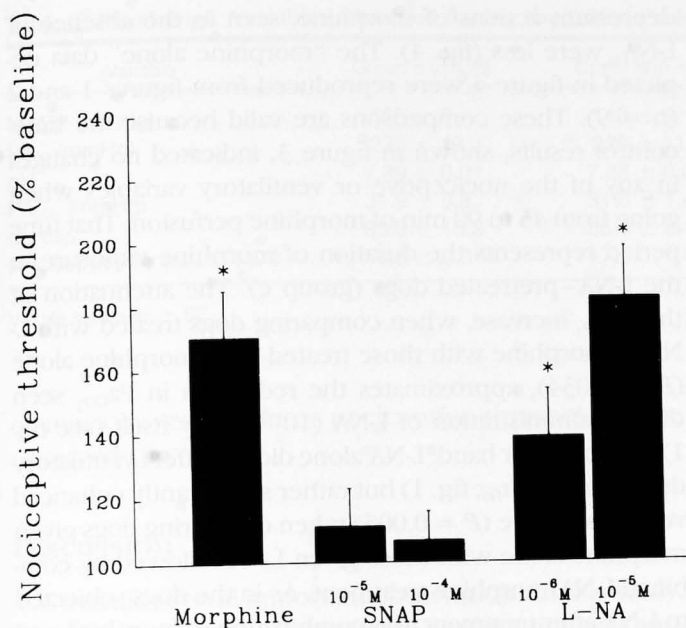


Fig. 2. The analgesic effects of fourth ventricular infusions of morphine (1  $\mu\text{g}/\text{ml}$ ;  $n = 9$ , S-nitroso-acetylpenicillamine (SNAP;  $10^{-5}$  and  $10^{-4}$  M;  $n = 7$ ), or nitro-L-arginine (L-NA;  $10^{-8}$  and  $10^{-5}$  M;  $n = 8$ ). The values (means  $\pm$  SE) are expressed as a percentage of the baseline nociceptive threshold (see table 2). \* $P < 0.05$  versus baseline.

pected, produced ventilatory depression (fig. 1). This is shown by the increased  $\text{Pa}_{\text{CO}_2}$  ( $\Delta\text{Pa}_{\text{CO}_2} = 7.4$  mmHg;  $P < 0.001$ ) and markedly reduced ventilatory drive ( $\text{dp}/\text{dt}_{60} = 52\%$  of baseline;  $P = 0.001$ ). Morphine, also as expected, had a significant antinociceptive effect (fig. 2). Thus morphine increased the nociceptive threshold to 170% of the baseline value ( $P < 0.001$ ). Nitro-L-arginine, the NO synthase inhibitor, appeared to elicit a modest dose-dependent stimulation of ventilation (fig. 1). This was manifested as a reduction in  $\text{Pa}_{\text{CO}_2}$  at the larger dose ( $P = 0.008$ ), but no significant change in ventilatory drive was seen. The data also indicated that L-NA was antinociceptive, with significant increases in nociceptive thresholds being observed at both the smaller (139% of baseline,  $P = 0.010$ ) and the larger dose (182% of baseline,  $P = 0.004$ ) of L-NA. The NO donor, SNAP, did not significantly affect ventilation (fig. 1), nor did it alter nociceptive threshold (fig. 2).

#### Effects of L-NA Posttreatment on Morphine-induced Actions

Effects of L-NA posttreatment on morphine-induced actions are summarized in figure 3, with baseline values given in table 2. When the dogs were given morphine,

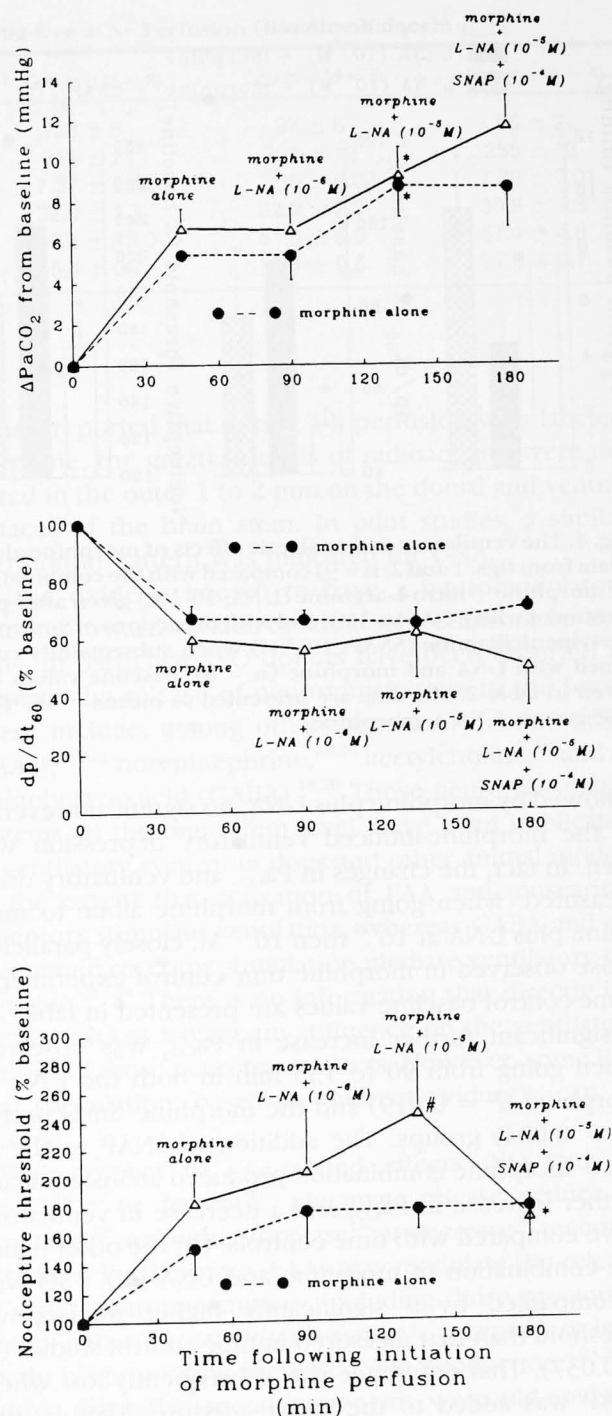


Fig. 3. The influence of morphine (1  $\mu\text{g}/\text{ml}$ ), followed by morphine + nitro-L-arginine (L-NA;  $10^{-6}$  and  $10^{-5}$  M), and then morphine + L-NA ( $10^{-5}$  M) + S-nitroso-acetylpenicillamine (SNAP;  $10^{-4}$  M) ( $n = 5$ ) on  $\text{Pa}_{\text{CO}_2}$  (top; expressed as the change from baseline), on  $\text{dp}/\text{dt}_{60}$  (middle; expressed as percentage of baseline), and on nociceptive threshold (bottom; expressed as percentage of baseline). As a control, the time-dependent effects of continuous morphine perfusion ( $n = 4$ ) are also presented. Values are means  $\pm$  SE. \* $P < 0.05$  versus baseline value (see table 2); # $P < 0.05$  versus morphine alone.

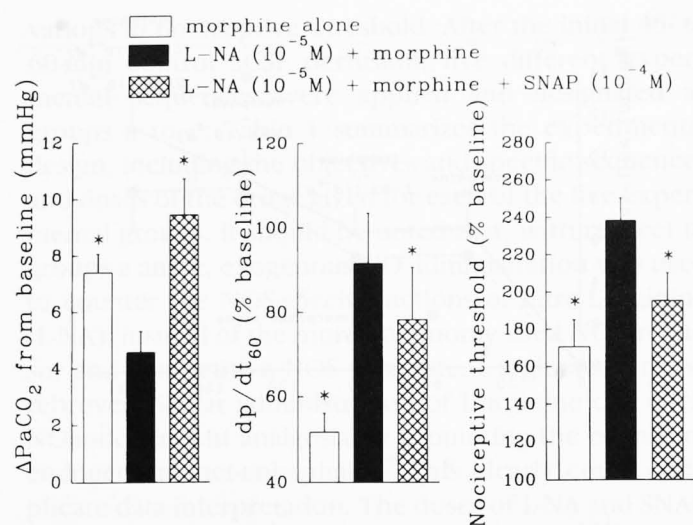


Fig. 4. The ventilatory and analgesic effects of morphine alone (data from figs. 1 and 2;  $n = 9$ ) compared with the combination of morphine + nitro-L-arginine (L-NA;  $10^{-5}$  M) given after pretreatment with L-NA ( $10^{-5}$  M); and with the actions of S-nitroso-acetylpenicillamine (SNAP;  $10^{-4}$  M) when subsequently combined with L-NA and morphine ( $n = 8$ ). Baseline values are given in table 2. The data are presented as means  $\pm$  SE. \* $P < 0.05$  versus L-NA + morphine.

followed by morphine plus L-NA, no significant reversal of the morphine-induced ventilatory depression was seen. In fact, the changes in PaCO<sub>2</sub> and ventilatory drive measured, when going from morphine alone to morphine plus L-NA at  $10^{-6}$  then  $10^{-5}$  M, closely paralleled those observed in morphine time-control experiments (time-control baseline values are presented in table 2). A significant further increase in PaCO<sub>2</sub> was observed when going from 90 to 135 min in both the L-NA + morphine ( $P = 0.019$ ) and the morphine time-control ( $P = 0.004$ ) groups. The addition of SNAP to the L-NA + morphine combination produced an insignificant further increase in PaCO<sub>2</sub> and a decrease in ventilatory drive compared with time controls. On the other hand, the combination of morphine and L-NA ( $10^{-5}$  M) was accompanied by a significantly higher nociceptive threshold than that measured in time-control studies ( $P = 0.037$ ). That difference was subsequently lost when SNAP was added to the above mixture. That is, the addition of SNAP reduced the nociceptive threshold from ~240% to ~180% of baseline ( $P = 0.025$ ).

#### Effects of L-NA before Treatment on Morphine-induced Actions

When we pretreated with L-NA and then administered morphine along with the higher L-NA dose, the ventilatory

depressant actions of morphine, seen in the absence of L-NA, were less (fig. 4). The "morphine alone" data depicted in figure 4 were reproduced from figures 1 and 2 ( $n = 9$ ). These comparisons are valid because the time-control results, shown in figure 3, indicated no changes in any of the nociceptive or ventilatory variables when going from 45 to 90 min of morphine perfusion. That time period represents the duration of morphine exposure in the L-NA-pretreated dogs (group c). The attenuation of the PaCO<sub>2</sub> increase, when comparing dogs treated with L-NA + morphine with those treated with morphine alone ( $P = 0.034$ ), approximates the reduction in PaCO<sub>2</sub> seen during administration of L-NA ( $10^{-5}$  M) by itself (see fig. 1). On the other hand, L-NA alone did not affect ventilatory drive (*i.e.*, dp/dt<sub>60</sub>; fig. 1) but rather significantly enhanced ventilatory drive ( $P = 0.005$ ) when comparing dogs given morphine alone with those given L-NA followed by combined L-NA/morphine treatment. As in the dogs subjected to L-NA after treatment, the combination of morphine and L-NA was associated with a significantly higher nociceptive threshold ( $P = 0.013$ ) than that seen with morphine alone (fig. 4). The addition of the NO donor, SNAP, to the L-NA/morphine mixture partially or completely reversed the effects of L-NA on PaCO<sub>2</sub> ( $P = 0.010$ ), dp/dt<sub>60</sub> ( $P = 0.004$  vs.  $10^{-5}$  M L-NA;  $P = 0.059$  vs. morphine alone), and nociceptive threshold ( $P = 0.002$ ).

#### Effects of Exogenous Nitric Oxide on the Ventilatory and Analgesic Actions of Morphine

When SNAP was combined with morphine, after a period of perfusion with morphine alone, no further

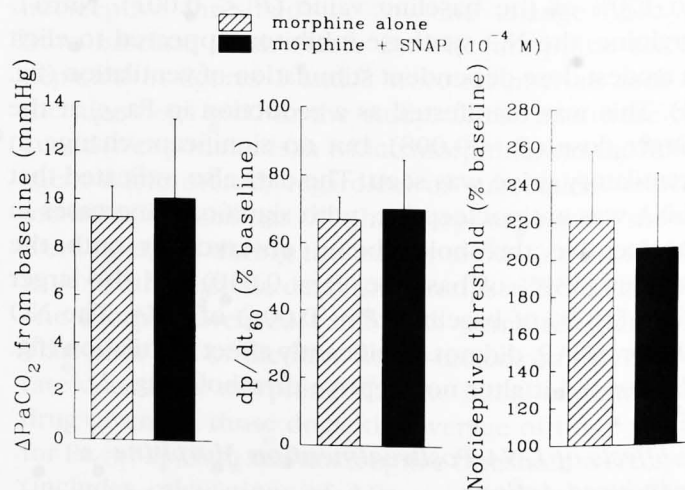


Fig. 5. The ventilatory and analgesic effects of morphine alone, followed by the combination of morphine + S-nitroso-acetylpenicillamine (SNAP;  $10^{-4}$  M;  $n = 5$ ). Baseline values are given in table 2. Values are means  $\pm$  SE.



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Table 2. Physiologic Variables Obtained during the Initial Period of Drug-free aCSF Perfusion (Baseline Values)

Variable	Group a (n = 5)	Group b (n = 7)	Group c (n = 8)	Group d (n = 5)	Group e (n = 4)
MABP (mmHg)	106 ± 6	110 ± 6	108 ± 5	97 ± 6	96 ± 2
PaO <sub>2</sub> (mmHg)	222 ± 19	229 ± 11	211 ± 21	221 ± 19	255 ± 13
pH	7.39 ± 0.01	7.38 ± 0.02	7.37 ± 0.02	7.36 ± 0.02	7.38 ± 0.01
PaCO <sub>2</sub> (mmHg)	31.9 ± 0.8	31.6 ± 1.4	32.9 ± 1.7	32.9 ± 2.2	30.9 ± 1.3
dp/dt <sub>60</sub>	83.9 ± 15.3	56.5 ± 8.4	68.4 ± 13.0	61.5 ± 6.0	57.4 ± 4.6
Nociceptive threshold (mA)	6.3 ± 0.3	7.1 ± 0.5	5.4 ± 0.7	7.1 ± 0.5	7.8 ± 0.7

Values are mean ± SE.

changes in ventilation or nociceptive thresholds were observed (fig. 5).

## Discussion

We made several important observations in this study. First, inhibition of endogenous NO production was antinociceptive and, under some circumstances, increased ventilatory drive. Second, exogenous NO did not affect ventilation or nociception, except when endogenous NO production had been suppressed. Third, NOS inhibition opposed the ventilatory depressant actions of morphine when given before, but not after, morphine administration. Fourth, regardless of the order of drug delivery, the combination of morphine and L-NA produced greater levels of analgesia than did morphine alone.

The possibility that NO may modulate ventilation has received very little attention in the literature. In rats and cats, NOS-positive neurons have been identified in medullary structures associated with ventilatory regulation and the effects of NOS inhibitors on ventilatory regulation have been examined.<sup>10,11</sup> Unfortunately, the results of those studies do not provide any clear indications of the ventilatory influence of NO. Furthermore, in addition to species-related factors, comparisons with present findings are complicated by, in the earlier studies, the use of anesthetics and by the fact that NOS inhibitors were administered either systemically<sup>10</sup> or by microinjection into the brain stem.<sup>11</sup> Thus the results of those investigations may be of little value when trying to explain the findings of our study.

The ventilatory influences of NO we observed are probably neural in nature and primarily involve superficial brain-stem structures. The latter contention is based on both published<sup>1</sup> and unpublished results from our laboratory concerning the intracerebral distribution of drugs delivered into the fourth ventricle. Thus we pre-

viously reported that during 1-h perfusion with labeled morphine, the greatest levels of radioactivity were detected in the outer 1 to 2 mm on the dorsal and ventral surfaces of the brain stem. In pilot studies, a similar distribution pattern was indicated for L-NA.

Nitric oxide is known to have a neuromodulatory function toward various cerebral neurotransmitter systems. In particular, NO donors have been reported to regulate the release of neurotransmitters in the brain. These include, among others, excitatory amino acids (EAAs),<sup>18-20</sup> norepinephrine,<sup>18,20</sup> acetylcholine,<sup>18</sup> and  $\gamma$ -aminobutyric acid (GABA).<sup>18,20</sup> Those neurotransmitter systems, at the brain-stem level, have been implicated in ventilatory control in dogs and other animal models to the extent that activation of EAA and muscarinic receptors stimulate ventilation, whereas GABA and  $\alpha_2$ -adrenergic receptor stimulation mediate ventilatory depression.<sup>21-23</sup> There is no information that directly implicates NO as having any influence on the ventilatory actions of those neurotransmitters. However, some limited speculation, based on indirect evidence, can be offered.

With respect to EAA-related effects, NO may increase<sup>18-20</sup> or decrease<sup>24</sup> glutamate release, reduce its reuptake,<sup>25</sup> and either increase<sup>26</sup> or decrease<sup>27</sup> receptor activities. Furthermore, EAAs may modulate the release of other neurotransmitters, including those previously noted, either directly *via* presynaptic receptors or indirectly by promoting increased NO production.<sup>4,23,28,29</sup> Within that rather broad framework, we could envision a scheme in which NO may act in association with EAA systems in producing the ventilatory effects that we saw. On the other hand, the literature indicates that if NO has any influence on the activity of muscarinic pathways it is by promoting acetylcholine release.<sup>18</sup> In light of the ventilatory stimulation that accompanies activation of brain-stem muscarinic receptors,<sup>21</sup> it seems unlikely that the ventilatory changes that we observed

could, in any direct way, be attributed to muscarinic cholinergic pathways. Further work is clearly needed before any conclusions can be made regarding the participation of EAA and cholinergic pathways in the ventilatory changes accompanying NOS inhibition and NO repletion.

Noradrenergic and GABAergic participation remain intriguing possibilities for several reasons. First, intracerebroventricular administration of GABA or the  $\alpha_2$ -adrenoceptor agonist, clonidine, was shown to depress ventilation in dogs.<sup>21,30</sup> Second, intracerebral administration of NO donors in rats is known to enhance norepinephrine and GABA release in multiple brain structures.<sup>18,31,32</sup> Third, NO was reported to activate  $\alpha_2$ -adrenoceptors in the brain-stem (presumably by promoting release of norepinephrine).<sup>32</sup> Fourth, NOS-positive and GABAergic neurons are found to colocalize in various cerebral structures, including the medulla.<sup>33-35</sup> Finally, we cannot ignore the possibility that our findings may be a function of a mechanism that involves an interplay among EAA and noradrenergic or GABAergic systems and NO, as suggested by the results of recent reports.<sup>23,28,29</sup> Studies investigating the potential contributions from EAA,  $\alpha_2$ -adrenergic, and GABA receptors in the ventilatory (and nociceptive) actions of NO inhibitors and donors are being planned in our laboratory.

Whatever mechanisms were involved in the ventilatory effects associated with NOS inhibition or exogenous NO in this study, the changes produced were modest and occurred under a limited set of conditions. Thus we observed a moderate enhancement of ventilation when L-NA was given to unpremedicated animals, and the level of ventilatory depression normally seen with morphine was lessened by L-NA given before but not after morphine treatment. The NO donor, SNAP, did not have any effect on ventilation unless endogenous NO production had been suppressed. This could still be consistent with a ventilatory depressant action of NO, but the sites involved in that action may be saturated under basal conditions. Thus only when endogenous NO levels were reduced could exogenous NO affect ventilation.

We cannot explain why L-NA lessened the ventilatory depressant effects of morphine before treatment but not after treatment. The pretreatment effect appeared to exhibit both additive and interactive components. That is, the  $\Delta Pa_{CO_2}$  in the dogs given L-NA, followed by L-NA + morphine was 3 to 4 mmHg less than that in dogs given morphine alone. That difference approxi-

mated the decrease in  $Pa_{CO_2}$  seen in the presence of L-NA alone, which suggests an additive effect. On the other hand, L-NA by itself did not alter  $dp/dt_{60}$ , but it did prevent the substantial reduction in  $dp/dt_{60}$  (relative to baseline) accompanying morphine administration, thus implying an interactive phenomenon.

Before proceeding to a discussion of the nociceptive changes observed in this investigation, it is important to emphasize that NOS inhibition permitted a substantial enhancement in the antinociceptive actions of morphine, without increasing, and in some instances diminishing, ventilatory depression. The major implication of those observations is that if we combine NOS inhibition with morphine, at any given level of analgesia, ventilatory depression will be less.

We developed the present model of nociception, paw withdrawal from electrocutaneous stimulation, specifically for use in unanesthetized dogs. Unlike in rodents, in large animals there are relatively few published methods for nociceptive assessments. However, electrocutaneous stimulation has been shown to be a valid and sensitive technique for evaluating nociceptive thresholds in various species, including rodents, nonhuman primates, and humans.<sup>36-38</sup> In human studies, electrocutaneous stimulation, compared with heat, was reported to be a more sensitive technique for assessing pain responsiveness.<sup>38</sup> Escape responses in rodents and monkeys compared favorably, although not precisely, with thresholds for pain sensation in humans, when similar current intensities were applied (see Vierck and co-workers<sup>37</sup>). This technique in monkeys has also proved sensitive to the antinociceptive actions of morphine.<sup>36</sup> That sensitivity to morphine was clearly shown in the present study. Using a single nociceptive paradigm does not permit us to generalize to all nociceptive pathways. For example, subdermal electrocutaneous stimulation presumably activates the more rapidly responding myelinated (probably A $\delta$ ), but not the slower-response unmyelinated (*i.e.*, C-type) afferents, which are thought to be activated by thermal stimuli.<sup>38,39</sup>

Nitro-L-arginine clearly was associated with enhanced antinociception, suggesting that endogenous NO production, in regions of the brain accessible to fourth ventricular administration, participates in the process of nociception in the dog. Unlike the ventilatory effects accompanying L-NA administration, nociceptive thresholds were consistently elevated in the presence of L-NA. This occurred regardless of whether L-NA was given alone or administered before or after morphine perfusions were begun. The observation that SNAP re-

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versed the antinociceptive effects associated with L-NA also can be taken as corroborative evidence that the L-NA-induced increase in morphine-associated analgesia was due to removal of NO.

Previous studies, performed in rodents, addressing the role of NO in nociceptive processing mediated at supraspinal sites, have yielded mixed results. This includes findings indicating no role for NO,<sup>40</sup> a nociceptive function for NO,<sup>5,7,9</sup> and an antinociceptive role for NO.<sup>8</sup> In the spinal cord, NO appears to have a critical function in nociceptive processing in rodent NMDA-dependent hyperalgesia models, to the extent that intrathecal NOS inhibition is antinociceptive.<sup>14,15</sup> However, under normal (nonhyperalgesic) conditions, intrathecal administration of NOS inhibitors may<sup>9</sup> or may not<sup>15,16</sup> affect nociception.

Several reports exist in the literature regarding NO-related effects on morphine-induced analgesia. Przewlocki and associates<sup>9</sup> reported that L-NAME potentiated morphine-induced analgesia at both spinal<sup>13</sup> and supraspinal<sup>9</sup> sites. On the other hand, Xu and Tseng<sup>41</sup> reported no effect of intracerebroventricular administration of the NO precursor, L-arginine, on intracerebroventricular morphine-induced analgesia. Similarly, Brigola and colleagues<sup>40</sup> reported no effect of intracerebroventricular L-arginine on the analgesia elicited by morphine injected subcutaneously. In that study, the morphine effect was attenuated by peripheral administration of L-arginine, an action reversed by peripheral injections of NOS inhibitors. However, caution must be observed when trying to interpret the results of studies using L-arginine administration. L-arginine is a substrate for brain tissue enzymes in addition to NOS. Thus L-arginine can be metabolized, through kyotorphin synthase, to kyotorphin, an endogenous enkephalin-releasing substance,<sup>7</sup> or through arginine decarboxylase, to agmatine.<sup>42</sup> Both of those substances promote antinociception.<sup>7,42</sup> Reports to date suggest that NO, at least at peripheral (presumably spinal) sites, opposes the antinociceptive effects of morphine. The literature is less clear concerning supraspinally mediated influences of NO on morphine-induced analgesia.

The effects we observed clearly represent actions at supraspinal sites (see Pelligrino and colleagues<sup>1</sup>). The results showed that the level of antinociception was greater in the presence of L-NA and morphine rather than morphine alone. We cannot label the effect of L-NA, when combined with morphine, as a potentiation of morphine-induced antinociception. The NOS inhibitor significantly increased the nociceptive threshold in

the absence of morphine. The greater antinociceptive effect seen with coprefusion of the two agents, as opposed to the levels seen when these agents were given separately, is probably best described as an additive effect. This suggests that morphine and L-NA act at separate sites to promote antinociception. The specific pathways influenced by NO in modulating nociception cannot be determined by the results obtained in this study. Nevertheless, it may be worthwhile to focus on those systems known to be influenced by NO. That is, EAA, noradrenergic, muscarinic, GABAergic, and serotonergic pathways all have been implicated in nociceptive processing<sup>16,43-46</sup> and warrant serious consideration in future studies.

Drugs administered *via* the present fourth ventricular delivery system essentially only gain access to superficial pontomedullary and periaqueductal gray (PAG) structures.<sup>1</sup> That distribution may be particularly important when trying to relate the effects of NO and opioid receptor activation to nociception. Periaqueductal gray tissue contains a fairly high density of opioid receptor ( $\mu$  and  $\delta$ )-expressing neurons and is an important structure in mediating the supraspinal antinociceptive actions of morphine.<sup>45,46</sup> In fact, PAG structures appear to be the site of origin of neurons that mediate supraspinal opioid-induced analgesia. Those neurons project to the rostral ventral medulla (RVM), an area also replete with opioid receptor-containing neurons<sup>47</sup> and some NOS-positive neurons as well.<sup>35</sup> The RVM neurons may then synapse with other brain-stem neurons that ultimately project to the spinal cord.<sup>45-47</sup> Fairly substantial nicotinamide adenine dinucleotide phosphate diaphorase staining (indicating the presence of NOS) has been identified in rat PAG.<sup>48</sup> Furthermore, microinjection of NO donors into the PAG elicited a marked inhibition of neuronal firing.<sup>48</sup> This is relevant because the PAG is also rich in GABAergic neurons, which may act to repress activity in the pathways connecting the PAG to the RVM.<sup>46</sup> Opioids, like morphine, acting on PAG receptors, are thought to produce antinociception by inhibiting GABAergic neurons, thus enhancing activity along the PAG-RVM pathway.<sup>46</sup> Blockade by GABA receptors within the RVM may also promote antinociception.<sup>49</sup> If we combine that information with reports showing that NO promotes GABA release in the brain,<sup>20</sup> then we could envision a mechanism whereby NOS inhibitors could be antinociceptive, simply by reducing GABA release in the PAG and RVM. That suggested NO-induced effect could be additive with an

action of morphine to reduce GABAergic neuron activity and therefore be in accord with present findings.

On the other hand, activation of muscarinic receptors in the rat RVM produces antinociception—an effect that is inhibited by L-NA.<sup>16</sup> This result would seem to run contrary to our findings. That disparity may simply be related to species differences or to the use of different nociceptive models. Nevertheless, these interesting observations emphasize the need to perform studies, using our dog model, that are designed to address the roles of GABAergic, muscarinic, and other systems in NO modulation of nociception at supraspinal sites. Studies concerning GABAergic mechanisms are particularly intriguing, because GABA at the brain-stem level promotes ventilatory depression (described previously). Thus, in one group of future experiments, receptor subtype-specific GABA blockers should be studied to determine if they can mimic the results we observed using L-NA or whether the SNAP-induced reversal of the antinociceptive and ventilatory actions of L-NA can be mimicked by GABA agonists. Noradrenergic pathways merit consideration in this regard. However,  $\alpha_2$ -adrenergic-related mechanisms do not seem to provide a likely explanation for the ventilatory and nociceptive changes we observed. That is, the diminished brain-stem  $\alpha_2$ -adrenergic activity that may accompany NOS inhibition, as described before, could explain the increase in ventilation we observed. Yet  $\alpha_2$ -agonists are also antinociceptive at supraspinal sites,<sup>50</sup> which would be contrary to the results we obtained here. The possibility remains that other adrenergic receptors might be involved. That issue can only be resolved by additional experiments.

Both NO and the  $\mu$ -agonist morphine affect nociception and ventilation. The results of our investigation indicate that inhibition of endogenous NO production can promote antinociception and, to a limited degree, increase ventilatory drive. Furthermore, NOS inhibition can add to the level of analgesia that accompanies morphine administration while also limiting morphine-induced ventilatory depression. The information that we gathered in this investigation may help to guide future approaches to pain management. In particular, NOS inhibitor pretreatment, followed by morphine, may permit the use of safer yet more effective analgesic doses of morphine.

The authors thank Richard Ripper for skillfully performing all surgical procedures and Anthony Sharp and David Visintine for their expert technical assistance.

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