Published in final edited form as:

Eur J Pharmacol. 2010 January 25; 626(2-3): 234–238. doi:10.1016/j.ejphar.2009.09.059.

Antagonism of the Antinociceptive Effect of Nitrous Oxide by Inhibition of Enzyme Activity or Expression of Neuronal Nitric Oxide Synthase in the Mouse Brain and Spinal Cord

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Abstract

Previous studies have implicated nitric oxide (NO) in the antinociceptive response to the anesthetic gas nitrous oxide (N₂O). The present study was conducted to confirm this NO involvement using pharmacological and gene knockdown and knockout strategies to inhibit the supraspinal and spinal production of NO. Antinociceptive responsiveness to 70% N₂O was assessed using the acetic acid (0.6%) abdominal constriction test in NIH Swiss mice following intracerebroventricular (i.c.v.) or intrathecal (i.t.) pretreatment with the NOS-inhibitor L-N^G-nitro arginine methyl ester (L-NAME) or an antisense oligodeoxynucleotide (AS-ODN) directed against neuronal NOS (nNOS). Experiments were also conducted in mice homozygous for a defective nNOS gene (nNOS^{-/-}). Mice that were pretreated i.c.v. or i.t. with L-NAME (1.0 µg) both exhibited 80-90% reduction in the magnitude of the N₂O-induced antinociceptive response. Mice that were pretreated i.c.v. or i.t. with nNOS AS-ODN (3×25 µg) exhibited a 60-80% antagonism of the antinociceptive response. Compared to wild-type mice, nNOS knockout mice showed a 60% reduction in N₂O-induced antinociception. These findings consistently demonstrate that transient or developmental suppression of nNOS expression significantly reduces antinociceptive responsiveness to N₂O. NO of both supraspinal and spinal origin, therefore, plays an important role in the antinociceptive response to N₂O.

Keywords

Nitrous oxide; Nitric oxide; Antinociception; NOS-inhibitor; Antisense; Transgenic mice

1. Introduction

Nitric oxide (NO) is an important central and peripheral signaling molecule and neurotransmitter. The scientific literature is replete with a maddening dichotomy of NO

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modulation of neurological, pathophysiological and psychological functions. For example, NO is reported to cause both neurotoxicity and neuroprotection (Calabrese et al., 2007). NO also appears to have a dual role in the modulation of depression as well as anxiety (da Silva et al., 2000; Spiacchi et al., 2008). NO can exert both proconvulsant and anticonvulsant influences (Ferraro and Sardo, 2004). Studies have shown NO can either initiate or inhibit neurogenesis (Cardenas et al., 2005). The precise role of NO in pain is also uncertain as studies have indicated both pronociceptive and antinociceptive roles for NO.

Prima facie evidence of NO involvement in any physiological function, including the examples listed above, is attenuation of that effect by inhibition of NO production. A number of analogs of L-arginine were found to competitively inhibit NOS enzyme and interfere with the production of NO (Rees et al., 1990). There is evidence aplenty to show that NO plays an important role in nociceptive processing (Meller and Gebhart, 1993; Aley et al., 1998). Treatment with pharmacological inhibitors of NO synthase (NOS) enzyme alone can produce antinociception (Moore et al., 1990, 1991; Handy and Moore, 1998). On the other hand, there is also evidence that pretreatment with NOS-inhibitors can interfere with the effects of a diverse group of drugs with antinociceptive properties, including clonidine (Przesmycki et al., 1999), morphine (Pataki and Telegdy, 1998; Abacıoğlu et al., 2001), [D-Pen²,D-Pen⁵]-enkephalin (Chen and Pan, 2003), sildenafil (Patil et al., 2004), and muscarinic agonists (Iwamoto and Marion, 1994a,b). Further, several studies have demonstrated that administration of NO donors can induce antinociception (Ferreira et al., 1992; Ji and Zhu, 1993; Chung et al., 2006) or enhance the antinociceptive effect of other drugs (Xu et al., 1995; Jain et al., 2001).

Our laboratory has long been interested in the role of NO in the antinociceptive effect of the anesthetic gas nitrous oxide (N₂O). Pursuant to Berkowitz' hypothesis that N₂O indirectly interacts with opioid receptors to produce antinociception in animals (Berkowitz et al., 1979), we provided the first chemical evidence that N₂O could induce neuronal release of endogenous opioid peptides (Quock et al., 1985). We also demonstrated a critical involvement of the biological regulator NO through antagonism of N₂O-induced antinociception by NOS-inhibitors (McDonald et al., 1994).

Previous investigations have demonstrated that inhibition of supraspinal NO synthesis can interfere with N₂O-induced antinociception in mice. The present study was conducted to administer identical doses of a NOS-inhibitor or an antisense oligodeoxynucleotide against neuronal NOS directly into the lateral cerebral ventricle or spinal cord and compare their efficacies of these routes of administration in antagonizing the antinociceptive response to N₂O.

2. Material and Methods

2.1. Animals

Male NIH Swiss mice, weighing 18-22 g, were purchased from Harlan Laboratories (Indianapolis, IN). Male mice homozygous for a defective nNOS gene (nNOS^{-/-}) and their wild-type (nNOS^{+/+}) counterparts, 18-22 g, were purchased from Charles River Laboratories (Charles River, MA). This study was approved by an institutional animal care and use committee with post-approval review and carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). All measures to minimize pain or discomfort were taken by the investigators.

Mice were housed five per cage in the Wegner Hall Vivarium at Washington State University with access to food and water ad libitum. The facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), was maintained on a 12-h light:dark cycle (lights on 07:00-19:00 h) under standard conditions (22 ± 1°C room

temperature, 33% humidity). Mice were kept in the holding room for at least four days after arrival in the facility for acclimation prior to experimentation.

2.2. Exposure to N₂O

N₂O and oxygen (O₂) (both medical grade, A-L Compressed Gases, Spokane, WA) were mixed and delivered using a dental-sedation system (Porter, Hatfield, PA) at a total flow rate of 10 L/min. Mice were individually exposed in a clear Plexiglas® exposure chamber (35 cm L × 20 cm W × 15 cm H) with gas inlet and outlet ports. The final gaseous mixture concentration of 70% N₂O and 30% O₂ in the box was verified using a POET II® anesthetic monitoring system (Criticare, Milwaukee, WI). The N₂O was based on concentration-response curves established in earlier studies (Quock et al., 1990; McDonald et al., 1994). Exhausted gases were routed by polyethylene tubing to a nearby fume hood. The total time that mice were exposed to N₂O was 11 min.

2.3. Antinociceptive Testing

Antinociceptive responsiveness was assessed using the abdominal constriction test (Siegmund et al., 1957). This nociceptive model was selected over tests employing a thermal noxious stimulus because this paradigm is significantly more sensitive to detection of κ opioid antinociceptive activity than are thermal tests (Tyers, 1980) and our previous studies have implicated κ opioid mechanisms in mediation of N₂O-induced antinociception (Quock et al., 1990). Mice were treated i.p. with 0.1 ml per 10 g body weight of 0.6% glacial acetic acid and placed into the Plexiglas® exposure chamber. Exactly 5 min later, the number of abdominal constrictions—lengthwise stretches of the torso with concave arching of the back—in each animal was counted for 6-min period for each treatment group while still in the N₂O atmosphere. Multiple raters were used for some but not all experiments; at least one of the raters was blinded to the drug treatment. All experiments were consistently conducted between 13:00 and 17:00 h. The control reference group was exposed to room air. The degree of antinociception (inhibition of abdominal constrictions) produced in various treatment groups of mice was calculated as:

$$\% \text{antinociception} = 100 \times \frac{\# \text{constrictions in control mice} - \# \text{constrictions in pretreated mice}}{\# \text{constrictions in control mice}}$$

2.4. Drugs

The following drugs were used in this research: L-N^G-nitro arginine methyl ester (L-NAME) (Research Biochemicals International, Natick, MA); and antisense or mismatch oligodeoxynucleotides (AS-ODN and MM-ODN, respectively) for neuronal NOS (Sigma Life Science, The Woodlands, TX). The 18-mer AS-ODN sequence (5'-GAA TCC TCT CCC CGC CCA-3') was designed to flank exon 18 within the mouse nNOS mRNA (nt 2815-2833, GenBank accession No. D14552) (Kolesnikov et al., 1997). The sequence 5'-GAA TCT CCT CCC GCC CCA-3' was used as the MM-ODN. Both AS- and MM-ODNs were designed as phosphorothioates to prevent degradation by endonucleases and were purified by RP-HPLC.

L-NAME was freshly prepared daily in sterile 0.9% physiological saline solution and administered at a dose of 1.0 μ g via intracerebroventricular (i.c.v.) or intrathecal (i.t.) routes 30 min prior to N₂O exposure. Preliminary studies showed that this dose of L-NAME alone had no antinociceptive effect. Control animals received i.c.v. or i.t. microinjections of vehicle (physiological saline solution); since microinjections of vehicle alone had no effect on N₂O-induced antinociception, i.c.v. and i.t. vehicle pretreatments were pooled for the control group indicated in the figures. AS- and MM-ODNs were dissolved in sterile 0.9% physiological saline

solution immediately prior to i.c.v. or i.t. microinjection. The dose of AS- and MM-ODNs for both i.c.v. and i.t. routes was 25 µg. For the i.c.v. microinjections, the ODNs were administered on alternative sides of the brain on days 1, 3 and 5. A separate control group received the same pattern of i.c.v. or i.t. microinjections of vehicle on days 1, 3 and 5.

2.5. Central Microinjection Procedures

I.c.v. pretreatments were made using the microinjection technique of Haley and McCormick (1958). Briefly, mice were anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL). A short incision was made along the midline of the scalp using a scalpel, and the skin was pulled back to expose the calvarium. The i.c.v. microinjection was made using a 10-µl microsyringe (Hamilton, Reno, NV) with a 26-gauge cemented needle. The microsyringe was held vertically by hand at a point on the calvarium 2.0 mm lateral and 1.0 mm caudal from bregma to a depth of -2.0 mm from the skull surface. Penetration was controlled by a large-bore needle through which the microsyringe needle was inserted through a large-bore hypodermic needle which served as a collar to limit penetration of the microsyringe needle to 2.0 mm. A volume of 5.0 µl of drug solution was delivered directly into the lateral cerebral ventricle over 30 sec.

The i.t. pretreatments were made using the microinjection technique of Hylden and Wilcox (1980). Briefly, mice were anesthetized with isoflurane. The mouse was held by the pelvic girdle and the microinjection was made through the skin into the spinal cord. A ½-inch, 30-gauge disposable needle attached to a 10-µl luer-tipped microsyringe (Hamilton) was inserted between the lumbar vertebrae below the L6 level (at the start of the cauda equina). A volume of 5.0 µl of drug solution was delivered directly into the spinal cord over 30 sec.

2.6. Statistical Analysis of Data

A one- or two-way ANOVA (as appropriate) with a post-hoc Bonferroni test was used to compare N₂O-induced antinociception in various treatment groups. Percent changes in antinociception were arcsine-transformed prior to statistical analysis.

3. Results

3.1. Influence of i.c.v. and i.t. NOS-inhibitor pretreatment on N₂O-induced antinociception

Exposure to 70% N₂O in O₂ resulted in a $91.4 \pm 1.9\%$ antinociceptive effect (Fig. 1). I.c.v. pretreatment with 1.0 µg L-NAME 30 min prior to N₂O exposure caused a 90% reduction in the antinociceptive response. Similarly, i.t. pretreatment with 1.0 µg L-NAME 30 min prior to N₂O exposure reduced the antinociceptive response by 85%.

3.2. Influence of i.c.v. and i.t. nNOS AS-ODN pretreatment on N₂O-induced antinociception

Neither i.c.v. nor i.t. pretreatment with 25 µg MM-ODN appreciably altered the magnitude of N₂O-induced antinociception (Fig. 2). The three i.c.v. pretreatments with 25 µg AS-ODN each caused a 75% reduction in the antinociceptive response to N₂O. Three i.t. pretreatments with 25 µg AS-ODN similarly caused a 55% reduction in the antinociceptive effect of N₂O.

3.3. Responsiveness of nNOS knockout and wild-type mice to N₂O-induced antinociception

Compared to NIH Swiss control mice, wild-type mice exhibited a $77.0 \pm 8.4\%$ antinociceptive response to 70% N₂O, while nNOS knockout mice exhibited a $22.9 \pm 6.4\%$ antinociceptive response (Fig. 3). Both responses were significantly less than that of the control group. The response of the nNOS knockout mice was 70% less than that of the wild-type group.

4. Discussion

Based on earlier research, there is evidence that the role of NO in pain is excitatory or inhibitory or both. Discrepancies in experimental findings were initially explained based on differences in drug selectivity, drug dosing or nociceptive testing procedures. However, the actual reasons appear to be much more complicated (Schmidtke et al., 2009).

A variety of inhibitors of NOS enzyme have been shown to induce an antinociceptive effect in several different models of experimental pain, including formalin-induced paw-licking, acetic acid-induced abdominal constriction and hot plate (Moore et al., 1991), carrageenan-induced hyperalgesia (Handy and Moore, 1998) and sciatic nerve-constriction-induced hyperalgesia (Lui and Lee, 2004).

Such findings suggested that the role of NO in pain is pronociceptive, likely in spinal nociceptive processing (Meller and Gebhart, 1993; Aley et al., 1998). Moreover, NOS-inhibitors also enhanced the antinociceptive effect of morphine (Machelska et al., 1997).

On the other hand, the results of other research suggested that NO was inhibitory to pain. Pretreatment with NOS-inhibitors reportedly antagonized the antinociceptive effects of morphine (Pataki and Telegdy, 1998; Abacioglu et al., 2001), opioid peptide derivatives (Chen and Pan, 2003) and muscarinic agonists (Iwamoto and Marion, 1994a,b) among others. Further, studies have demonstrated an analgesic or antinociceptive response to NO donors (Ferreira et al., 1992; Ji and Zhu, 1993; Chung et al., 2006). NO-donors also enhanced the antinociceptive effect of other drugs such as morphine (Lauretti et al., 2002), sufentanil (Lauretti et al., 1999), β -endorphin (Xu et al., 1995) and sildenafil (Jain et al., 2001). More recent studies, however, have reported biphasic, dose-related effects of NO donors on pain (Sousa and Prado, 2001; Prado et al., 2002).

Previously we reported that N₂O-induced antinociceptive effects in the mouse abdominal constriction test and the rat hot plate test were sensitive to antagonism by a series of L-arginine analogs, including L-NAME, L-N^G-nitro arginine (L-NOARG) and L-NMMA, L-N^G-monomethyl nitro arginine (L-NMMA) (McDonald et al., 1994). The antagonism was reversed by co-administration of the NOS substrate L-arginine together with the inhibitor; co-administration of D-arginine, which is not a substrate for NOS, was ineffectual in reversing the antagonism.

In the present study, three approaches were utilized to interfere with the production of NO in the brain and spinal cord. L-NAME is a pharmacological inhibitor of the NOS enzyme (Rees et al., 1990). nNOS AS-ODN prevents expression of the nNOS enzyme (Kolesnikov et al., 1997) and can cause significant reductions in regional brain nNOS mRNA (Kolesnikov et al., 1997) and NOS enzyme activity (Li et al., 2003). nNOS^{-/-} mice are homozygous for a defective nNOS gene and fail to express nNOS (Huang et al., 1993). All three approaches were effective in significantly reducing the magnitude of the antinociceptive response to N₂O.

The present results demonstrate that N₂O-induced antinociception is equally sensitive to antagonism by i.c.v. vs. i.t. pretreatment with the NOS-inhibitor L-NAME or an AS-ODN directed against nNOS. Therefore, it would seem that the NO that is involved in N₂O-induced antinociception may be both supraspinal as well as spinal.

In an earlier investigation, we posited that NO may be a regulator of stimulated neuronal release of endogenous opioid peptides (Hara et al., 1995). It had been demonstrated earlier that i.c.v. microinjection of β -endorphin stimulated the release of met-enkephalin in the spinal cord of intrathecally-perfused rats (Tseng et al., 1986). When L-NOARG was added to the artificial cerebrospinal fluid (aCSF) perfusate, the amount of met-enkephalin collected from the spinal

cord following microinjection of β -endorphin was reduced by 50%. When L-arginine but not D-arginine was added to the L-NAME in the aCSF perfusate, the amount of met-enkephalin released by β -endorphin was restored to control levels. These findings suggest that NO plays a role in the neuronal release of endogenous opioid peptides in the rat spinal cord.

We have amassed evidence that N_2O -induced antinociception is sensitive to antagonism by i.c.v. and i.t. pretreatment with norbinaltorphimine, a kappa opioid antagonist (Quock et al., 1990) as well as rabbit antisera against rat dynorphin (Branda et al., 2000; Cahill et al., 2000). Combined with the results of this study which demonstrates antagonism of N_2O -induced antinociception by i.c.v. and i.t. pretreatment with L-NAME and antisense against neuronal NOS, we hypothesize that N_2O -induced antinociception in the mouse abdominal constriction test is secondary to an NO-dependent stimulated neuronal release of dynorphin that subsequently activates kappa opioid receptors in the brain and spinal cord.

5. Conclusion

While the precise role of NO in pain remains uncertain, the results of this research clearly demonstrate that NO is critical to the antinociceptive response of mice to N_2O . The drug effect was significantly attenuated or even abolished in mice following pretreatment with a pharmacological inhibitor of NOS or antisense oligodeoxynucleotide directed against nNOS and in mice homozygous for a defective nNOS gene. The sites of NO-dependent opioid peptide release appear to be both supraspinal and spinal.

Acknowledgments

This research was supported by NIH Grants DA-10047 (R.M.Q.) and GM-77153 (R.M.Q.) and funds from the WSU College of Pharmacy.

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Fig. 1. Influence of i.c.v. and i.t. NOS-inhibitor pretreatment (1.0 $\mu\text{g}/\text{mouse}$) on N_2O -induced antinociception. The data are expressed as the mean $\pm$ S.E.M. of 8-12 mice per group. Significance of difference: ***, $P < 0.001$, compared to the vehicle + N_2O treatment group (one-way ANOVA and post-hoc Bonferroni's multiple comparison test).

Fig. 2.

Influence of i.c.v. and i.t. nNOS AS-ODN pretreatment ($3 \times 25 \mu\text{g}$) on N_2O -induced antinociception. The data are expressed as the mean $\pm$ S.E.M. of 8-12 mice per group. Significance of difference: ***, $P < 0.001$, compared to the MM-ODN-pretreated group by the respective route (two-way ANOVA and post-hoc Bonferroni's multiple comparison test).

Fig. 3. Responsiveness of nNOS^{+/+} (wild-type) mice and nNOS^{-/-} (knockout) mice to N₂O-induced antinociception. The data are expressed as the mean ± S.E.M. of 10-15 mice per group. Significance of difference: ***, $P < 0.001$, compared to wild-type mice (one-way ANOVA and post-hoc Bonferroni's multiple comparison test).