Role of Nitric-Oxide Synthase Isoforms in Nitrous Oxide Antinociception in Mice

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ABSTRACT

Exposure of mice to the anesthetic gas N₂O evokes a prominent antinociceptive effect that is sensitive to antagonism by nonselective nitric-oxide synthase (NOS) inhibitors. The present study was conducted to identify whether a specific NOS isoform is implicated in N₂O antinociception in mice. In the abdominal constriction test, exposure of mice to 25, 50, and 70% N₂O resulted in a concentration-dependent antinociceptive effect that persisted for up to 6 min following removal of the mice from the N₂O atmosphere into room air. This N₂O antinociceptive effect was antagonized by pretreatment with S-methyl-L-thiocitrulline (SMTC) and higher doses of L-N²-(1-iminoethylo)ornithine (L-NIO), which reportedly inhibit the neuronal and endothelial isoforms of NOS, respectively. Nevertheless, the N₂O-induced antinociception was unaffected by pretreatment with low doses of either SMTC or L-NIO or by pretreatment with 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), which selectively inhibits inducible NOS. The s.c. pretreatment with SMTC and L-NIO reduced brain NOS activity in a dose-dependent manner, whereas AMT had no such effect. Moreover, in blood pressure experiments, SMTC increased SBP in a dose-unrelated fashion, whereas L-NIO showed an appreciably weaker but dose-related increase in SBP. The i.c.v. pretreatment with SMTC also reduced N₂O antinociception and brain NOS activity without increasing SBP. These results suggest that it is the neuronal isoform of NOS that is involved in mediation of the antinociceptive effect of N₂O in the mice.

N₂O is a widely used general anesthetic gas with many clinical applications, including enhancement of basal anesthesia by an inhalation anesthetic agent (i.e., the second gas effect) (Evers and Crowder, 2001), production of conscious sedation for dental surgery in anxious patients (Jackson and Johnson, 2002), and emergency relief of severe anxiety and pain (Kennedy and Luhmann, 2001). N₂O has also been used for patient-administered analgesia (Castera et al., 2001), relief of labor pains (Rosen, 2002), pre-emptive analgesia (Katz, 1995), and reduction of pain and discomfort in various medical procedures, including intra-articular drug injection (Cleary et al., 2002), peripheral intravenous cannulation (Gerhardt et al., 2001), sigmoidoscopy (Harding and Gibson, 2000), colonoscopy (Forbes and Collins, 2000), ophthalmologic procedures (Cook et al., 2002), and biopsy procedures (Masood et al., 2002).

An involvement of endogenous opioid systems in N₂O-induced analgesia is evidenced by observations that N₂O antinociception in experimental animals was sensitive to antagonism by naloxone and other opioid receptor blockers (Berkowitz et al., 1976; Quock et al., 1990, 1993). There is also evidence that N₂O antinociception is secondary to stimulated neuronal release of endogenous opioid peptides (Quock et al., 1985; Zuniga et al., 1987).

Previous studies in our laboratory using the mouse abdominal constriction model have demonstrated that N₂O antinociception was antagonized in a dose-related fashion by naloxone (Quock et al., 1993) and, more specifically, by selective κ-opioid receptor blockers (Quock et al., 1990). This was also verified by the failure of β-chlornaltrexamine to antagonize N₂O antinociception in mice, in which κ-opioid receptors were protected against aklylation by coadministration of a κ-opioid ligand (Quock and Mueller, 1991). This implication of κ-opioid receptors is also consistent with recent reports that N₂O antinociception in mice is antagonized by i.c.v. and i.t. pretreatment with rabbit antisera against rat dynorphin (Branda et al., 2000; Cahill et al., 2000).

Previous findings from our laboratory also demonstrated that inhibition of NO synthesis antagonized N₂O antinociception in mice, in which NOS activity without increasing of SBP. These results suggest that it is the neuronal isoform of NOS that is involved in mediation of the antinociceptive effect of N₂O in the mice.

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ABBREVIATIONS: NO, nitric oxide; NOS, nitric-oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; SMTC, S-methyl-L-thiocitrulline; L-NIO, L-N²-(1-iminoethylo)ornithine; AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; SBP, systolic blood pressure; L-NAME, L-N⁴-nitro arginine methyl ester; L-NOARG, L-N⁴-nitro arginine; U.S.P., United States Pharmacopeia.
ception in rats and mice (McDonald et al., 1994). NOS inhibitors also attenuated the ability of i.c.v. administered β-endorphin to stimulate the neuronal release of methionine-enkephalin in the rat spinal cord (Hara et al., 1995), suggesting that stimulated neuronal release of endogenous opioid peptides might be dependent on NO.

NO is synthesized as a by-product of conversion of its physiological precursor L-arginine to L-citrulline. This reaction is catalyzed by a family of enzymes known as NO synthase (NOS). There are two constitutive forms of the enzyme—neuronal NOS (nNOS) and endothelial NOS (eNOS)—and an inducible form, inducible NOS (iNOS). nNOS is classically found in the central and peripheral neurons where NO plays a role in neurotransmission and neuromodulation. eNOS is largely found in endothelial cells and has a substantial role in blood pressure regulation (Dominiczak and Bohr, 1995). These two NOS isoforms are regulated by Ca²⁺ and calmodulin and are constitutively expressed in tissues. In contrast, iNOS is widely distributed among immune cells, including macrophages and glial cells, is induced by various stimuli (e.g., endotoxin), and is activated independent of Ca²⁺ (Jacobs et al., 1997). The recent development of compounds that possess relative selectivity for inhibiting different isoforms of NOS allows identification of the specific NOS isoforms involved in specific physiological, pathological, or pharmacological functions.

The aim of the present study was to use isoform-selective NOS inhibitors and determine whether their influences on N₂O antinociception were consistent with an involvement of the neuronal form of NOS.

Materials and Methods

Animals. Male NIH Swiss mice (20–33g) were purchased from Harlan Sprague-Dawley Laboratories (Indianapolis, Indiana) and used in this research. Mice were housed five per cage, with food and water available ad libitum in the American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited Wegner Hall Vivarium at Washington State University. The facility was maintained on a 12 h light/dark cycle (lights on 7:00 AM to 7:00 PM) under standard conditions (22°C room temperature, 33% humidity). Mice were kept in the holding room for at least 4 days following arrival in the facility. Animals were used only one time then discarded. This research was approved by the institutional animal care and use committee of Washington State University and was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Antinociceptive Testing in Mice. Antinociception was assessed using the abdominal constriction test. The mice were treated i.p. with 0.1 ml/10 g b.wt. of 0.6% glacial acetic acid in distilled water and immediately placed into the test chamber; exactly 5 min later, the number of abdominal contractions—length-wise stretches of the torso with concave arching of the back—was counted for each mouse over a 6-min observation period. In prior training sessions, the numbers of abdominal contractions recorded in test animals were very consistent between trained observers who were or were not aware of drug treatment. Consequently, the observer for the experiments in this study was not blind to the drug condition of the various groups of mice.

Groups of one to six mice each were exposed to N₂O inside an enclosed prefilled Plexiglas box (35 cm long × 20 cm wide × 15 cm high) with an airtight hinged lid. N₂O in O₂ was continuously delivered into the box using a standard dental sedation system (Porter, Hatfield, Pennsylvania). The amounts of N₂O and O₂ were varied within a total inflow rate of 10 l/min to achieve the desired test concentration (25% N₂O: 2.5 l/min N₂O and 7.5 l/min O₂; 50% N₂O: 5.0 l/min N₂O and 5.0 l/min O₂; and 70% N₂O: 7.0 l/min N₂O and 3.0 l/min O₂). Gas entered the box through an inflow port at one end, circulated through the box, and exited through an outflow port at the other end. Exhausted gases were vented from the box to a nearby fume hood. The concentrations of N₂O and O₂ in the box were continuously monitored using a POET II anesthetic monitoring system (Criticare, Milwaukee, Wisconsin). Control animals were exposed to room air in lieu of N₂O and O₂.

In most experiments of this study, the protocol consisted of an i.p. injection of acetic acid followed by a 5-min exposure to N₂O in the chamber then removal from the exposure chamber to a cage in room air, followed by a 6-min observation period, during which the number of abdominal contractions was recorded. In room air-exposed mice, acetic acid-induced abdominal contractions generally appear in 2 to 3 min, peak during the 6-min observation period, and slowly ebb with occasional contractions occurring beyond 6 min.

To verify that the antinociceptive effect of N₂O was still in effect during the 6-min observation time, three additional groups of mice were tested under modified conditions. As represented in the left panel of Fig. 1, the atmosphere during the 6-min observation period was varied from all N₂O to all room air with two intermediate states wherein mice were transferred from N₂O to room air 2 and 4 min into the 6-min observation period.

The antinociceptive effect of N₂O in different treatment groups of mice was quantified using the following formula: % antinociception = 100 × (no. contractions in control mice − no. in control mice in exposed mice)/[no. contractions in control mice). Separate vehicle-treated groups of mice were used as controls.

Assay of Neuronal Nitric-Oxide Synthase Activity. Mice were pretreated s.c. or i.c.v. with different doses of each NOS inhibitor. After pretreatment times of 30 min for S-methyl-L-thiocitrulline (SMTcO) and N⁵-(1-iminoethyl)-L-ornithine (L-NIO) and 60 min for 2-amino-5,6-dihydroxy-6-methyl-4H-1,3-thiazine (AMT), the mice were euthanized by decapitation for collection of cerebella following s.c. pretreatment or whole brains following i.c.v. pretreatment. NOS activity was determined in the cerebellar or brain homogenate by the conversion of [¹⁴C]L-arginine to [¹⁴C]L-citrulline. The cerebellum or whole brain was homogenized in 2 volumes of Tris-HCl buffer (50 mM, pH 7.4), containing 2 mM EDTA and 2 mM EGTA, and centrifuged at 12,000 rpm at 4°C for 5 min. Twenty microliters of supernatant were added to test tubes containing 50 mM Tris-HCl buffer, 10 mM NADPH, 6 mM CaCl₂, 62 mM tetrahydrobiopterin, 2 μM flavin adenine dinucleotide, 2 mM flavin mononucleotide, and 0.5 μCi [¹⁴C]L-arginine monohydrochloride (Amersham Biosciences, Piscataway, NJ) in a final volume of 40 μl at pH 7.4. Following incubation at 37°C for 30 min, the reaction was terminated by the addition of 50 mM HEPES buffer containing 5 mM EDTA and resin. Then the reaction mixture was applied onto 1.5-mL columns of Dowex
AG50WX-8 (Bio-Rad, Hercules, CA). [14C]L-citrulline was quantified by scintillation spectroscopy of 10-ml aliquots of the flow through. The protein concentration was determined using a standard protein assay kit (Pierce Chemical Company, Rockford, Illinois). NOS activity was expressed in terms of picomoles of citrulline formed per milligram of protein per minute and then expressed as a percentage of control.

**Measurement of Systolic Blood Pressures.** Systolic blood pressure measurements were made noninvasively by the plethysmographic (tail-cuff) technique, using a model 59 pulse amplifier and dual channel recorder (IITC, Inc., Life Science, Woodland Hills, CA). Mice were anesthetized with an i.p. injection of ketamine (150 mg/kg) and xylazine (12.5 mg/kg). The mouse tail was inserted through the inflatable cuff of the sensor block, which also contained the photoelectric sensor and light source. The cuff was inflated to occlude the tail blood supply. As the pressure was slowly released, a sensitive pulse transducer detected the return of blood flow, and the "breakthrough" SBP was determined from the strip chart. The tail-cuff measures were derived from the average of three measurements per animal. In NOS inhibitor-treated mice, mice received either s.c. or i.c.v. injections of SMTC or L-NIO after a resting SBP was established (which generally required 15 min). After a 30-min pretreatment time, SBPs were determined and compared with the resting SBP.

**Drugs.** The following drugs were used in this research: N2O, U.S.P., and O2, U.S.P. (A and L Welding, Spokane, Washington), bregma and to a depth of 2.5 mm from the skull surface. The point on the calvarium 1.0 mm lateral to and 2.0 mm caudal to the AD50 values and 95% confidence intervals were determined and compared by the method of Litchfield and Wilcoxon (1949). In the chemical experiments, the significance of difference between treatment groups was determined by one-way analysis of variance and a post hoc Tukey test.

**Results**

**N2O Antinociception in Mice.** A 5-min exposure of mice to 70% N2O caused a uniform reduction in the number of abdominal constrictions regardless of whether the N2O exposure was terminated (i.e., mice were removed from the N2O chamber and transferred to a cage in room air) immediately after the 5-min exposure period, 2 min into the 6-min assessment period, 4 min into the 6-min assessment period, or throughout the 6-min assessment period. Figure 1 shows that the level of antinociception is constant regardless of the duration of N2O exposure.

**Influence of Isoform-Selective NOS Inhibitors on N2O Antinociception in Mice.** The s.c. pretreatment with 10, 30, or 50 mg/kg SMTC, the nNOS inhibitor, antagonized N2O antinociception in a dose-related manner, as indicated by a progressive rightward shift of the N2O antinociception dose-response curve (Fig. 2). The i.c.v. pretreatment with 1.0 μg SMTC/mouse also significantly attenuated N2O antinociception (Fig. 3). The s.c. pretreatment with 10 or 30 mg/kg L-NIO, the eNOS inhibitor, resulted in antagonism of N2O antinociception only at the higher dose (Fig. 4). The s.c. pretreatment with 1.0 mg/kg AMT, the iNOS inhibitor, failed to influence N2O antinociception (Fig. 5). None of these NOS inhibitors administered alone suppressed abdominal constrictions.

Table 1 compares the AD50 values for N2O antinociception in the various treatment groups depicted in Figs. 2 through 5. There is a significant increase in the AD50 values for N2O antinociception in treatment groups receiving any of two s.c. doses of SMTC, the i.c.v. dose of SMTC, or the higher dose of L-NIO. Conversely, there is no appreciable change in the AD50 values for N2O antinociception in treatment groups receiving AMT or the lower dose of L-NIO.

**Influence of NOS Inhibitors on Cerebellar NOS Activity in Mice.** The s.c. pretreatment with increasing doses...
of SMTC and L-NIO resulted in a significant and dose-dependent reduction of cerebellar NOS activity (Fig. 6). On the other hand, there was no significant effect of AMT on cerebellar NOS activity. The i.c.v. pretreatment with 1.0 μg of SMTC/mouse also significantly reduced whole-brain NOS activity. The i.c.v. pretreatment with 1.0 mg/kg of L-NIO caused a dose-dependent increase in the mean SBP.

**Discussion**

There is abundant evidence that NO participates in neurotransmission or a signal transduction role in the central nervous system. Previous studies have demonstrated that pharmacological manipulation of brain NO levels can have significant impact on a variety of neurologic and behavioral functions, including neuropeptidergic (Kiss, 2000), neuroendocrine regulation (Rivier, 2001), synaptic plasticity (Holscher, 1997), behavior (McLeod et al., 2001), thermoregulation (Kamerman et al., 2000), food intake (Kamerman et al., 1995; Tracey et al., 1995). On the other hand, AMT is a selective inhibitor of iNOS, being 10-fold more potent against iNOS than nNOS and 42-fold more potent against eNOS than iNOS (Rees et al., 1990; McCall et al., 1991). AMT is a selective inhibitor of iNOS, being 10-fold more potent against iNOS than nNOS and 42-fold more potent against eNOS than iNOS (Rees et al., 1990; McDonald et al., 1994). L-NIO is approximately 8-fold more potent against eNOS than nNOS and 4-fold more potent against eNOS than iNOS (Furfine et al., 1994). L-NIO is approximately 8-fold more potent against eNOS than nNOS and 4-fold more potent against eNOS than iNOS (Furfine et al., 1994).

**Table 2**

The influence of pretreatment with NOS-inhibitors on SBP in mice

<table>
<thead>
<tr>
<th>Dose</th>
<th>Mean Systolic Blood Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0 min</strong></td>
<td><strong>30 min</strong></td>
</tr>
<tr>
<td>Vehicle</td>
<td>66.5 ± 9.7</td>
</tr>
<tr>
<td>SMTC (10 mg/kg, s.c.)</td>
<td>63.5 ± 10.7</td>
</tr>
<tr>
<td>SMTC (30 mg/kg, s.c.)</td>
<td>67.5 ± 6.7</td>
</tr>
<tr>
<td>SMTC (50 mg/kg, s.c.)</td>
<td>67.6 ± 2.3</td>
</tr>
<tr>
<td>SMTC (1.0 μg/mouse, i.c.v.)</td>
<td>66.7 ± 5.1</td>
</tr>
<tr>
<td>1-NIO (10 mg/kg, s.c.)</td>
<td>65.1 ± 7.3</td>
</tr>
<tr>
<td>1-NIO (30 mg/kg, s.c.)</td>
<td>68.6 ± 9.5</td>
</tr>
</tbody>
</table>

Significance of difference: *, P < 0.05 versus vehicle control; †, P < 0.01 versus Vehicle; ‡, P < 0.01 versus 1-NIO (10 mg/kg).
nNOS, the question is whether the eNOS of the cerebral Since most NO in the brain likely results from an action of lipopolysaccharide-induced increase in plasma nitrites and selectivity (Boer et al., 2000), it also had no effect on cerebel-
lar NOS activity at a dose that significantly reduced the high-dose L-NIO (30 mg/kg) antagonized N₂O antinociception and inhibited cerebellar NOS activity. This is likely the result of loss of selectivity for eNOS and emerging inhibition of nNOS at higher doses (30 mg/kg).

Secondly, we attempted to identify an appropriate index of eNOS activity. Attempts to measure NOS activity in de-
seding aorta were fraught with difficulty because of limited tissue mass and low levels of NOS activity. Since eNOS is largely found in endothelial cells and is thought to have a substantial role in blood pressure regulation (Dominiczak and Bohr, 1995), it was thought that SBP might be sensitive to changes in eNOS activity. It is known that inhibition of eNOS can induce an increase in SBP (Rees et al., 1990). In the present study, both SMTCT and L-NIO elevated mean SBP. Although SMTCT was previously characterized as a se-
ctive inhibitor of the nNOS isoform (Furfine et al., 1994), all three doses of SMTCT significantly increased the mean SBP, which was consistent with earlier reports that SMTCT elevates SBP (Narayanan et al., 1995). This effect has been explained on the basis of possible inhibition of nNOS in cardiovascular-regulating regions of the brain (Ollerstam et al., 1997), but detailed mechanisms remain unclear. These SBP-increasing effects of SMTCT were not dose-related and were not correlated to antagonism of N₂O antinociception in a dose-dependent manner as did inhibition of cerebellar NOS activity.

In the present study, L-NIO caused significant dose-related increases in the mean SBP. This SBP-elevating effect of L-NIO was weaker than that of SMTCT, however. Although previous reports showed that L-NIO increased SBP significantly, that effect was 10 times weaker than the other argi-
nine analog inhibitors (L-NAME or L-NOARG) (Rees et al., 1990). In our preliminary experiments, the SMTCT effect was comparable to that of L-NOARG. Therefore, the present results are consistent with the results of earlier studies.

In addition to experiments using systemic pretreatment with NOS inhibitors, SMTCT was also introduced directly into the brain. SMTCT, administered via the i.c.v. route, produced a significant antagonism of N₂O antinociception. This pre-
treatment also significantly cerebellar NOS activity without causing an elevation in mean SBP. In earlier research, L-
NAME, a nonselective NOS inhibitor, was also found to be an effective antagonist of N₂O antinociception following i.c.v. pretreatment (McDonald et al., 1994).

In conclusion, N₂O antinociception in the mouse abdominal constriction test was most effectively antagonized by the selective nNOS inhibitor SMTCT in a dose-dependent manner. This antagonism of antinociception was dose dependently correlated with inhibition of cerebellar NOS activity but not with increasing SBP. At low doses, the selective eNOS inhib-
itor L-NIO was ineffective in antagonizing N₂O antinocicep-
tion or inhibiting cerebellar NOS activity. A higher dose of L-NIO not only reduced N₂O antinociception but also inhibited cerebellar NOS activity. L-NIO also caused a dose-related increase in SBP. It is presumed that, at high doses, L-NIO loses its selectivity for eNOS and also affects nNOS. The selective iNOS inhibitor AMT was ineffectual in antag-
onizing N₂O antinociception and also failed to reduce cere-
bellar NOS activity. These results suggest that the neuronal isoform of NOS is involved in mediation of the antinocice-
tive effect of N₂O in the mouse.

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