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Involvement of a NO–cyclic GMP–PKG signaling pathway in nitrous oxide-induced antinociception in mice

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Abstract

The antinociceptive effect of nitrous oxide (N₂O) is dependent on nitric oxide (NO); however, the next step in the pathway activated by NO is undetermined. The present study was conducted to test the hypothesis that a N₂O action involves sequential activation of NO synthase, soluble guanylyl cyclase and protein kinase G to induced an antinociceptive effect in mice. The antinociceptive responsiveness of male NIH Swiss mice to N₂O was assessed using the acetic acid abdominal constriction test. Different groups of mice were pretreated with either saline, the NO scavenger 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (carboxy-PTIO), the guanylyl cyclase-inhibitor (1H-[1,2,4]-oxadiazolo-[4,3-a]quinoxalin-1-one) (ODQ), the protein kinase G-inhibitor Rp-isomer of 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphorothioate (Rp-8-pCPT-cGMPS) or the selective phosphodiesterase V-inhibitor 1,2-dihydro-2-[(2-methyl-4-pyridinyl)methyl]-1-oxo-8-(2-pyrimidinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride (T 0156). Vehicle (saline)-pretreated mice responded to N₂O in a concentration-dependent manner. This antinociceptive effect was antagonized by systemic pretreatment with carboxy-PTIO and ODQ and central pretreatment with Rp-8-pCPT-cGMPS. In each case, the dose-response curve for N₂O was progressively shifted to the right by increasing doses of each pretreatment drug. On the other hand, N₂O-induced antinociception was enhanced by systemic pretreatment with T 0156; the dose-response curve for N₂O was shifted to the left. The ATP-sensitive potassium channel blocker glibenclamide was without influence on the anti-nociceptive effect of N₂O. These results support the hypothesis that N₂O-induced antinociception in mice is mediated by a NO–cyclic GMP–PKG pathway.

Keywords

Nitrous oxide; antinociception; nitric oxide; cyclic GMP; protein kinase G; mice

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1. Introduction

Previously we implicated the biological regulator nitric oxide (NO) in the responsiveness of mice to the anesthetic gas nitrous oxide (N₂O) (Cope et al., 2010). Transient suppression of neuronal nitric oxide synthase (nNOS) expression by intracerebroventricular (i.c.v.) or intrathecal (i.t.) pretreatment with the NOS-inhibitor L-N^G-nitro arginine methyl ester or an antisense oligodeoxynucleotide directed against neuronal NOS (nNOS) both reduced the magnitude of the N₂O-induced antinociceptive response. Developmental suppression of nNOS in mice homozygous for a defective nNOS gene (nNOS^{-/-}) also blunted the magnitude of the antinociceptive response to N₂O, when compared to wild-type (nNOS^{+/+}) mice.

A variety of studies have shown that a common signal transduction pathway activated by NO involves the stimulation of soluble guanylyl cyclase (sGC), which then increases intracellular levels of the second messenger 3',5'-cyclic guanosine monophosphate (cyclic GMP) (Southam and Garthwaite, 1993; Denninger and Marletta, 1999). The NO signal can then be passed downstream to a variety of signaling targets, which may include cyclic GMP-dependent protein kinases (PKG) (Ota et al., 2008), cyclic GMP-gated cation channels (Barnstable et al., 2004) and cyclic GMP-regulated phosphodiesterases (PDE) (Lin et al., 2010). This study will test the hypothesis that the antinociceptive effect of N₂O is mediated by a signaling pathway, which involves NO, cyclic GMP and protein kinase G. A pharmacological approach was utilized to evaluate the role of each of these components of the signaling pathway.

2. Material and Methods

2.1. Animals

Male NIH Swiss mice, weighing 18–22 g, were purchased from Harlan Laboratories (Indianapolis, IN) and used in these experiments, which were 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 Animal Resource Unit 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 Nitrous Oxide (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). 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; Quock and Mueller, 1991). Mice were treated i.p. with 0.1 ml per 10 g body weight of 1.0% glacial acetic acid in deionized distilled water and placed into the 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 while under N₂O. 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 generally conducted between 0900 and 1200 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: 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide, potassium salt (carboxy-PTIO), dihydro-2-[(2-methyl-4-pyridinyl)methyl]-1-oxo-8-(2-pyrimidinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride (T 0156) and glibenclamide (Tocris Bioscience, Ellisville, MO); 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) (Axxora LLC, San Diego, CA); Rp-8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphorothioate, sodium salt (Rp-8-pCPT-cGMPS) (Enzo Life Sciences, Plymouth Meeting, PA). All pretreatments were administered 30 min prior to testing.

Carboxy-PTIO was dissolved in bacteriostatic saline and was administered in i.p. doses of 0.3 and 0.6 mg/kg. ODQ, T 0156 and glibenclamide were dissolved in dimethyl-sulfoxide (DMSO), and distilled water was added to attain a final DMSO concentration of 1%. ODQ was administered i.p. in doses of 1.0 and 3.0 mg/kg, and glibenclamide was likewise administered i.p. in doses of 1.0, 3.0 and 10 mg/kg. T 0156 was administered in an i.p. dose of 20 mg/kg. The volume of i.p. injection was 0.1 ml/10 g body weight. The respective vehicles were administered to control animals.

Rp-8-pCPT-cGMPS was dissolved in distilled water and administered in i.c.v. doses of 0.3 and 1.0 μ l in a volume of 4.0 μ l via the intracerebroventricular (i.c.v.) route according to the method of Haley and McCormick (1958). Mice were anesthetized with IsoFlo® (isoflurane, U.S.P., 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- μ l 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; the larger hypodermic needle served as a collar to limit penetration of the microsyringe needle to 2.0 mm. A volume of 4.0 μ l of drug solution was delivered directly into the lateral cerebral ventricle over 30 s.

2.5. Statistical Analysis of Data

Dose-responses curves for N₂O-induced antinociception were constructed from the degree of suppression of acetic acid-induced abdominal constrictions by each concentration of N₂O in each treatment group. The probit of the percent antinociceptive response (percent inhibition of abdominal constrictions) was plotted against the log concentration of N₂O, and the antinociceptive dose, 50% (AD₅₀) values and 95% confidence intervals were determined by the method of Litchfield and Wilcoxon (1949), as described by Dewey et al. (1970). The glibenclamide data were analyzed using one-way analysis of variance (ANOVA) with a post-hoc Bonferroni multiple comparison test.

3. Results

3.1. N₂O-Induced Antinociception

An i.p. injection of 1.0% glacial acetic acid typically induced abdominal constrictions with an onset of 3–4 min and a duration of approximately 12–15 min. Within a 6-min window that commences 5 min after injection, mice exhibited an average of 16.3±1.0 abdominal constrictions (N=18). Following exposure to increasing concentrations of N₂O (25%, 50% and 70%), the mean number of glacial acetic acid-induced abdominal constrictions was reduced to 10.1±0.6 (37.8% antinociceptive effect), 5.8±2.4 (64.6% antinociceptive effect) and 1.3±0.7 (89.5% antinociceptive effect), respectively.

3.2. Effect of carboxy-PTIO on the antinociceptive activity of N₂O

In animals that were pretreated i.p. saline vehicle, exposure to 70% N₂O evoked an 89.5% antinociceptive effect. In mice that were pretreated with one of two doses of carboxy-PTIO (0.3 or 0.6 mg/kg) 30 min earlier, the peak antinociceptive effect was reduced in a dose-related manner to 67.0% and 56.4%, respectively. There was a rightward shift in the dose-response curve for N₂O-induced antinociception (Fig. 1).

Administration of carboxy-PTIO alone caused a slight reduction in the number of abdominal constrictions that was not statistically significant. The AD₅₀ value (with 95% confidence intervals) for N₂O-induced antinociception was 32.8% (20.1–53.6%) for the vehicle control group, whereas AD₅₀ value was increased to 47.1% (31.3–71.0%) in the 0.3 mg/kg treatment group and 62.1% (46.6–82.9%) in the 0.6 mg/kg treatment group. Only the AD₅₀ value from the higher carboxy-PTIO dose group was statistically significant from control ($P < 0.05$).

3.3. Effect of ODQ on the antinociceptive activity of N₂O

In another experiment, mice that were pretreated with two doses of ODQ 30 min beforehand responded to 70% N₂O with a dose-related reduction in the number of abdominal constrictions. The peak antinociceptive effect of 95.0% in mice pretreated with vehicle (1% DMSO) and exposed to 70% N₂O was reduced to 80.7% and 49.2% by pretreatment with 1.0 and 3.0 mg/kg ODQ, respectively. Comparison of dose-response curves in the absence and presence of ODQ show that inhibition of cyclic GMP production shifted the dose-response curve for N₂O-induced antinociception to the right (Fig. 2). The AD₅₀ value of the vehicle control group was 31.1% (20.4–47.3%), while those of the 1.0 and 3.0 mg/kg ODQ treatment groups increased to 42.0% (32.6–54.1%) and 74.5% (60.5–91.9%), respectively. Only the AD₅₀ value of the higher dose ODQ group was statistically significant from control ($P < 0.05$).

3.4. Effect of Rp-8-pCPT-cGMPS on the antinociceptive activity of N₂O

In mice that were pretreated i.c.v. with bacteriostatic saline, 25%, 50% and 70% N₂O produced 31.0%, 60.7% and 91.3% antinociceptive effects, respectively. I.c.v. pretreatment with 0.3 and 1.0 g/mouse Rp-8-pCPT-cGMPS reduced the antinociceptive response of different groups of mice to all three concentrations of N₂O. There was a progressive rightward shift in the dose-response curve for N₂O-induced antinociception in the presence of the two doses of Rp-8-pCPT-cGMPS (Fig. 3). The AD₅₀ value for N₂O-induced antinociception was 35.8% (23.5–54.2%) for the i.c.v. vehicle control group, while the AD₅₀ value was increased to 48.3% (33.6–69.4%) in the 0.3 µg treatment group and 80.3% (54.7–117.8%) in the 0.6 µg treatment group. Only the AD₅₀ value from the group receiving 1.0 µg/mouse of Rp-8-pCPT-cGMPS was statistically significant from the control group ($P < 0.05$).

3.5. Effect of T 0156 on the antinociceptive activity of N₂O

On the other hand, systemic pretreatment with 20 mg/kg T 0156 enhanced the antinociceptive response to all three concentrations of N₂O and caused a leftward shift in the dose-response curve (Fig. 4). The AD₅₀ value of vehicle control group was 34.4% (21.7–54.3%), and the AD₅₀ value of the T 0156 treatment group was decreased to 17.0% (10.5–27.4). The difference between these two AD₅₀ values was statistically significant ($P < 0.05$).

3.6. Effect of glibenclamide on the antinociceptive activity of N₂O

Different groups of mice were pretreated with three increasing doses of glibenclamide (1–10 mg/kg). These doses of glibenclamide had no appreciable effect on the antinociceptive effect of N₂O (Fig. 5).

4. Discussion

These findings clearly implicate the involvement of a NO–cyclic GMP–PKG pathway in the antinociceptive effect of N₂O in mice. Carboxy-PTIO is an NO scavenger that exhibits an inhibitory action against the free NO radical (Akaike et al., 1993). Its ability to antagonize N₂O-induced antinociception replicates the antagonism of N₂O by pharmacological NOS-inhibitors (McDonald et al., 1994; Ishikawa and Quock, 2003) and antisense oligodeoxynucleotides against nNOS (Li et al., 2004). ODQ is a highly selective, irreversible inhibitor of soluble guanylyl cyclase (Garthwaite et al., 1995). Its ability to antagonize N₂O-induced antinociception supports the contention that N₂O stimulates production of NO that stimulates the production of cyclic GMP. Rp-8-pCPT-cGMPS is an inhibitor of PKG or cyclic GMP-dependent protein kinase (Butt et al., 1994). Its ability to antagonize N₂O-induced antinociception suggests that the N₂O stimulates the production of cyclic GMP, which, in turn, acts upon PKG along the signaling pathway resulting in the antinociceptive response. T 0156 is a selective inhibitor of phosphodiesterase V, which is specific for cyclic GMP, and can prolong the cyclic GMP-mediated activation of PKG (Mochida et al., 2002). The observed potentiation of N₂O-induced antinociception by T 0156 is consistent with the idea that cyclic GMP is a key mediator of the antinociceptive effect.

There are studies that indicate that the ATP-sensitive potassium (K_{ATP}) channel may lie at the terminus of a NO–cyclic GMP–PKG pathway (Sachs et al., 2004; Vale et al., 2007; Wang et al., 2008). But the failure of glibenclamide, a K_{ATP} channel blocker (Ripoll et al., 2007), to alter the antinociceptive response to 70% N₂O even in doses up to 10 mg/kg suggests that the K_{ATP} channel may not be involved in N₂O-induced antinociception.

Carboxy-PTIO, ODQ and T 0156 pretreatments were administered systemically so their drug effects occur peripherally as well as centrally. Nonetheless, it is still likely that the NO–cyclic GMP–PKG pathway resides in the central nervous system. In previous research, N₂O-induced antinociception was antagonized in dose-related fashion by i.c.v. pretreatment with either arginine analogs that competitively inhibit nNOS (McDonald et al., 1994; Ishikawa and Quock, 2003) or antisense oligodeoxynucleotide directed against nNOS (Li et al., 2004). It is also consistent with the inhibitory effect of i.c.v.-administered Rp-8-pCPT-cGMPS in the present study.

Earlier we implicated the endogenous κ opioid ligand dynorphin and brain and spinal cord κ opioid receptors in N₂O-induced antinociception. The N₂O response was antagonized by i.c.v. and i.t. pretreatment with the selective κ opioid antagonist norbinal-torphimine (Quock et al., 1990) and rabbit antiserum against rat dynorphin (Branda et al., 2000; Cahill et al., 2000). How is this reconciled with the present findings that implicate an NO–cyclic GMP–PKG pathway in the antinociceptive activity of N₂O?

There are two possibilities. One is that the neuronal release of endogenous opioid peptide is NO-dependent, as suggested by the antagonism of release of spinal cord methionine-enkephalin by NOS-inhibitor (Hara et al., 1995) and that cyclic GMP and PKG are components of this presynaptic signaling pathway that regulates opioid peptide release. The feasibility of this concept is strong as NO has been implicated as a modulator in the neuronal release of several different neurotransmitters (Lonart et al., 1992; Prast and Philippu, 1992; Tanioka et al., 2002; Kodama and Koyama, 2006) and endocrine hormones (Uretsky et al., 2002; Rubinek et al., 2005).

The other possible explanation is that the signaling pathway activated by the κ opioid receptor may itself involve NO–cyclic GMP–PKG. In favor of this possibility is the reported activation of an NO/cyclic GMP pathway by the κ opioid agonist bremazocine (Amarante and Duarte, 2002); however, the bremazocine was administered locally in the paw amid a well-characterized NO–cyclic GMP–PKG–K_{ATP} pathway that mediates peripheral analgesia. The idea of a κ opioid receptor-activated NO–cyclic GMP–PKG pathway is mitigated by observations that NOS-inhibitors slightly potentiate or do not affect κ opioid receptor-mediated antinociception (McDonald et al., 1994; Machelska et al., 1997); the κ opioid agonist U-50,488H reduces cerebral cortical NOS enzyme activity (Barjavel and Bhargava, 1994); and U-50,488H-induced antinociception is not enhanced by L-arginine (Xu and Tseng, 1993).

It is interesting that a NO–cyclic GMP–PKG pathway is also involved in another pharmacological effect of N₂O, the relief of anxiety. The anxiolytic effect of N₂O in the mouse light/dark exploration test was significantly reduced by NOS-inhibitors and nNOS-directed antisense oligodeoxynucleotides (Li et al., 2003a, 2003b), ODQ (Li et al., 2004) and Rp-8-pCPT-cGMPS (Li et al., 2005).

5. Conclusion

The findings of this study support the hypothesis that a NO–cyclic GMP–PKG pathway is involved in N₂O-induced antinociception. This was successfully demonstrated by competitive antagonism of the antinociceptive effect of N₂O by inhibition of NO transit, inhibition of cyclic GMP production, and inhibition of the PKG enzyme. The location of this NO-cyclic GMP-PKG pathway—whether it is a presynaptic regulator of opioid peptide release or a post-opioid receptor signal transduction pathway—remains to be determined.

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Fig. 1. Effect of carboxy-PTIO on the antinociceptive effect of N₂O: ○, vehicle + N₂O (control); ●, 0.3 mg/kg carboxy-PTIO + N₂O; and □, 0.6 mg/kg carboxy-PTIO + N₂O. Each symbol represents the mean ± S.E.M. of at least 8 mice per group.

Fig. 2. Effect of ODQ on the antinociceptive effect of N₂O: ○, vehicle + N₂O (control); ●, 1.0 mg/kg ODQ + N₂O; and □, 3.0 mg/kg ODQ + N₂O. Each symbol represents the mean ± S.E.M. of at least 8 mice per group.

Fig. 3. Effect of Rp-8-pCPT-cGMPS on the antinociceptive effect of N₂O: ○, vehicle + N₂O (control); ●, 0.3 µg Rp-8-pCPT-cGMPS + N₂O; and □, 1.0 µg Rp-8-pCPT-cGMPS + N₂O. . Each symbol represents the mean ± S.E.M. of at least 8 mice per group.

Fig. 4. Effect of T 0156 on the antinociceptive effect of N₂O: ○, vehicle + N₂O (control); ●, 20 mg/kg T 0156 + N₂O. Each symbol represents the mean ± S.E.M. of at least 8 mice per group.

Fig. 5. Effect of glibenclamide on the antinociceptive effect of 70% N₂O. Each column represents the mean antinociceptive effect $\pm$ S.E.M. of at least 8 mice per group. There was no significant difference between any of the treatment groups.