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Role of Brain Dynorphin in Nitrous Oxide Antinociception in Mice

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BRANDA, E. M., J. T. RAMZA, F. J. CAHILL, L. F. TSENG AND R. M. QUOCK. *Role of brain dynorphin in nitrous oxide antinociception in mice.* PHARMACOL BIOCHEM BEHAV **65**(2) 217–221, 2000.—Earlier studies indicate that nitrous oxide antinociception is mediated by opioid receptors, and we have hypothesized that nitrous oxide stimulates a neuronal release of an endogenous opioid peptide (EOP) that stimulates opioid receptors. To further test this hypothesis, male NIH Swiss mice were pretreated intracerebroventricularly with rabbit antisera to opioid peptides or with various inhibitors of peptidases involved in the degradation of EOPs. Mice were subsequently exposed to three different concentrations of nitrous oxide in oxygen, and their antinociceptive responsiveness was measured using the acetic acid abdominal constriction test. Nitrous oxide antinociception was significantly attenuated by 24-h pretreatment with antisera to various fragments of dynorphin (DYN) but not by antisera against methionine-enkephalin (ME) or β-endorphin (β-EP). In other experiments, nitrous oxide antinociception was significantly enhanced by 30-min pretreatment with phosphoramidon, an inhibitor of endopeptidase 24.11, which has been implicated in DYN degradation, but not bestatin or captopril, which inhibit aminopeptidase and angiotensin-converting enzyme, respectively. The latter enzymes have been implicated in degradation of certain EOPs albeit not DYN. These findings support the hypothesis that nitrous oxide antinociception in the mouse abdominal constriction test is mediated by endogenous DYN acting in the central nervous system. © 2000 Elsevier Science Inc.

Nitrous oxide antinociception Opioid peptides Antisera to opioid peptides Peptidase inhibitors

RESEARCH in our laboratory has been directed at elucidating the opioid mechanism of action of nitrous oxide antinociception. Using the abdominal constriction test, we have previously reported that nitrous oxide evokes a concentration-related antinociceptive effect in mice that is sensitive to antagonism by opioid receptor blockers (21). We narrowed the field to κ-opioid receptors because nitrous oxide antinociception is specifically sensitive to antagonism by the κ-opioid receptor blocker norbinaltorphimine but not μ- or δ-opioid receptor blockers (20,24). It was originally hypothesized that nitrous oxide might owe its pharmacological action to stimulation of the neuronal release of opioid peptides in the brain (2). Accordingly, we speculate that nitrous oxide antinociception in mice might be due to stimulated release of dynorphin (DYN), which is a family of endogenous κ-opioid peptides (4,5). This hypothesis was tested by pharmacologically manipulating free levels of DYN and other opioid peptides in the brain, and determining the influence on nitrous oxide antinociception in mice.

METHOD

Animals

Male NIH Swiss mice, 18–25 g, were purchased from Harlan–Sprague–Dawley Laboratories (Indianapolis, IN).

Antinociceptive Testing

Antinociceptive responses were assessed using the abdominal constriction test 24 h following antiserum pretreatment or 30 min following peptidase-inhibitor pretreatment. Mice

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were treated intraperitoneally with 0.1 ml per 10 g body weight of 0.6% acetic acid; 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 a 6-min period. Multiple raters were used for some but not all experiments; the number of abdominal constrictions counted was very consistent between the raters. During their observations, the raters were unaware of the ICV pretreatment given to test animals, but were aware of the concentration of nitrous oxide being administered. The degree of antinociception (inhibition of abdominal constrictions) produced by nitrous oxide in various treatment groups of mice was calculated as:

$$\% \text{ antinociception} = 100 \times \frac{\text{Number of constrictions in control mice} - \text{Number of constrictions in exposed mice}}{\text{Number of constrictions in control mice}}$$

Drugs

The following drugs were used in this research: Nitrous Oxide, U.S.P., Oxygen, U.S.P. and Compressed Air, U.S.P. (Rockford Industrial Welding Supply, Rockford, IL); antisera to porcine dynorphin A 1–8 (DYN 1–8) and dynorphin A 1–13 (DYN 1–13) and phosphoramidon (Peninsula Laboratories, Belmont, CA); bestatin and captopril (Sigma Chemical Company, St. Louis, MO); and rabbit antisera to rat ME, human β-EP, camel β-EP, and control rabbit serum (prepared in Dr. Tseng's laboratory, as described below).

Nitrous oxide and oxygen were delivered into a Plexiglas exposure chamber (20 cm W × 35 cm L × 15 cm H) using a standard dental sedation system (Porter, Hatfield, PA). Proportions of nitrous oxide and oxygen were varied within a total inflow rate of 10 l/min to achieve the desired test concentrations of nitrous oxide (25, 50, and 70%). Concentrations of gases within the chamber were confirmed with a POET II anesthetic monitoring system (Criticare, Milwaukee, WI). Exhausted gases were vented to a fumehood. Control animals were exposed to Compressed Air, U.S.P. delivered into the chamber at the same inflow rate (10 l/min).

Antisera against porcine DYN 1–8 and DYN 1–13 were purchased from Peninsula Laboratories (Belmont, CA). The DYN 1–8 antiserum crossreacted 100% with porcine DYN 1–8, < 0.01% with various other large fragments of DYN, and not at all with various endorphins and enkephalins (Peninsula Laboratories product information). The DYN 1–13 antiserum crossreacted 100% with porcine DYN 1–17, porcine DYN 1–13, porcine big DYN 1–24 and porcine DYN 1–12, 25% with porcine big DYN, 1.5% with porcine DYN 1–11 and porcine DYN 1–10 amide, and not at all with porcine DYN 1–6 through 1–10, β-EP or ME (Peninsula Laboratories product information).

The antisera against β-EP and ME were produced in Dr. Tseng's laboratory by repeated injection of rabbits with human or camel β-EP and rat ME conjugated to bovine thyroglobulin (13). The antisera were collected, purified, characterized, lyophilized, and stored at −20°C. The β-EP antisera crossreacted 100% with β-lipotropin but not at all with ME, LE, various DYNs, β-melanotropin, or ACTH. The lyophilized antisera were dissolved in sterile saline before use. The ME antiserum crossreacted 100% with ME, 30% with ME-Lys, 12.7% with ME-Arg-Phe, <1% with ME-Arg-Gly-Leu, <1% with LE, <1% with LE-Arg, <1% with β-endorphin, <1% with DYN 1–8, <1% with DYN 1–10, and not at all with oxidized ME, DYN 1–13, DYN 1–17 or neurotensin. Doses of antisera reflect the dry weight (μg) of the lyophilized serum. Lyophilized rabbit serum was used in lieu of antisera for control experiments.

Optimal doses and pretreatment times for antisera to influence nitrous oxide antinociception were determined in preliminary experiments. Mice were anesthetized by a brief exposure to halothane, U.S.P. (Halocarbon, River Edge, NJ). Antisera and peptidase inhibitors were all prepared in physiological saline and injected ICV in a volume of 4.0 μl using a hand-held 10-μl glass microsyringe (Hamilton, Reno, NV) (11) 24 h or 30 min prior to nitrous oxide exposure, respectively. In control experiments, physiological saline was similarly administered ICV. These microinjections were consistently made unilaterally on the right side. In preliminary and earlier studies, ICV microinjection of a dye marker was performed to indicate successful placement of the microinjection into the right lateral cerebral ventricle.

Statistical Analysis of Data

Dose–response curves were constructed for nitrous oxide antinociception in different pretreatment groups. The probit of the percent antinociception (percent inhibition of abdominal constrictions) was plotted vs. log concentration of nitrous oxide, and the AD_{50} values with 95% confidence intervals were determined using the method of Litchfield and Wilcoxon (17) as described by Dewey et al. (8).

RESULTS

ICV pretreatment with 100 μg antisera against DYN 1–8 or DYN 1–13 significantly reduced the antinociceptive response of mice to all three concentrations of nitrous oxide, compared to responses of a control group pretreated ICV 24 h earlier with control serum. Lower ICV doses of these antisera were either ineffective (30 μg) or inconsistent (80 μg) in influencing the effects of nitrous oxide. Likewise, antisera pretreatment times of 1 and 2 h were not consistent in antagonizing nitrous oxide antinociception.

When the dose–response curves were plotted, the curves of the DYN antisera-pretreated groups were shifted to the right of that for the control group (Fig. 1). Similar ICV pretreatment with antisera to β-EP and ME had no apparent influence on the antinociceptive effect of nitrous oxide. Comparison of the AD_{50} values showed that the AD_{50} values of both DYN antisera pretreatment groups were significantly greater than that of the control group; the AD_{50} value of the β-EP and ME antisera pretreatment groups were not significantly different from that of the control group (Table 1).

Following ICV pretreatment with phosphoramidon, mice exhibited an enhanced antinociceptive response to nitrous oxide. The dose–response curve for nitrous oxide antinociception in the phosphoramidon-pretreated mice was shifted to the left of the control dose–response curve (Fig. 2). Comparable ICV pretreatment of mice with either bestatin or captopril had no apparent influence on nitrous oxide antinociception (Fig. 2). Comparison of the AD_{50} values showed the AD_{50} value of the phosphoramidon-pretreated group to be significantly less than that of the saline control group; on the other hand, the AD_{50} values of the bestatin and captopril groups were similar to that of the control group (Table 2).

DISCUSSION

In previous research, we have found that the antinociceptive effect of nitrous oxide in mice is sensitive to antagonism

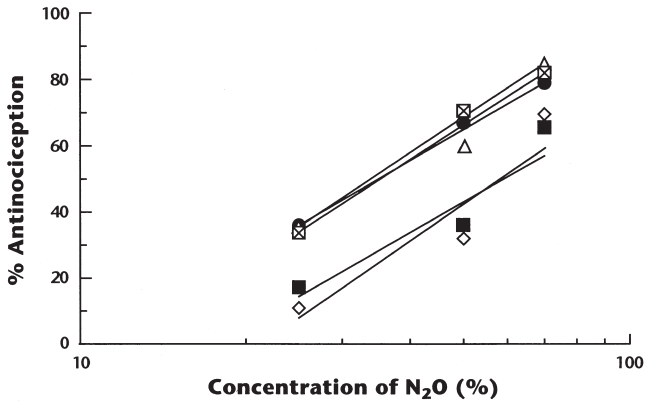

FIG. 1. Dose–response curves for nitrous oxide antinociception in mice following ICV pretreatment with: ☒, 100 μg control rabbit serum; ●, 100 μg β-EP AS; △, 100 μg ME AS; ■, 100 μg DYN 1–8 AS; and ◇, 100 μg DYN 1–13 AS. Each point represents the mean antinociceptive response of at least 10 mice.

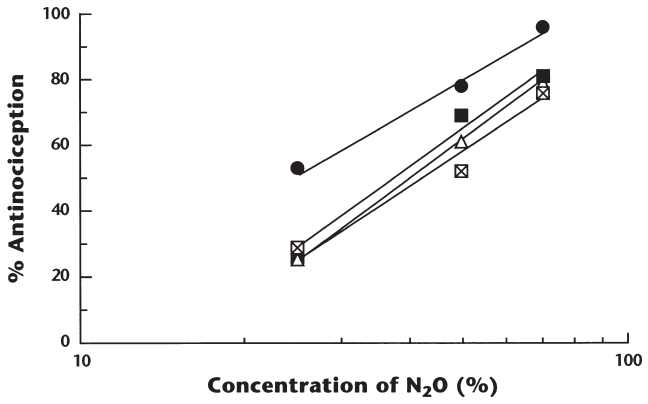

FIG. 2. Dose–response curves for nitrous oxide antinociception in mice following ICV pretreatment with: ☒, physiological saline; ●, 3.0 μg phosphoramidon; △, 10 μg bestatin; and ■, 300 ng captopril. Each point represents the mean antinociceptive response of at least 10 mice.

by the κ-opioid receptor blocker norbinaltorphimine but not by μ- or δ-opioid receptor blockers (20), and is protected from β-chlornaltrexamine antagonism by concurrent administration of U-50, 488H, a κ-opioid ligand (23). These findings strongly implicate κ-opioid receptors in the nitrous oxide-induced antinociception in mice (24). There is existing precedence for mediation of certain effects of nitrous oxide by κ-opioid receptors, as nitrous oxide reportedly generalizes to the discriminative cue produced by the κ-opioid agonist ethylketocyclazocine rather than to that of morphine (15). This is also supported by an early study in which nitrous oxide ameliorated withdrawal symptoms from the κ-opioid analgesic drug pentazocine (16).

If nitrous oxide acts indirectly on opioid receptors, as suggested by Berkowitz and associates (2), and demonstrated in rats (22,26,30), then one must conclude that the antinociceptive effects of nitrous oxide in the mouse abdominal constriction test must result from neuronal release of DYN because the DYNs are endogenous κ-opioid ligands (4,5). This is confirmed in the present study by the ability of ICV-administered DYN antisera but not β-EP or ME antisera to antagonize nitrous oxide antinociception. Because both DYN AS effectively attenuated the nitrous oxide response, the DYN 1–8 AS does not immunoreact with longer DYN fragments, and the DYN 1–13 AS does not immunoreact with shorter DYN fragments, it is suggested that nitrous oxide may be capable of releasing both long and short forms of DYN. Future studies might utilize combinations of these antisera as well as assess the effectiveness of antisera against α-neoendorphin or DYN B in influencing nitrous oxide antinociception.

Another point regarding antiserum antogonism of nitrous oxide antinociception is worthy of mention. Pretreatment with DYN 1–8 AS reduced the antinociceptive effect of 25% nitrous oxide by one-half but caused only a 20% reduction in the antinociceptive effect of 70% nitrous oxide. Similarly, pretreatment with DYN 1–13 AS reduced the antinociceptive effect of 25% nitrous oxide by two-thirds but caused only a 15% reduction in the antinociceptive effect of 70% nitrous oxide. The diminution of antagonism against higher concentrations of nitrous oxide might reflect additional mechanisms that may come into play at higher concentrations.

If nitrous oxide stimulates neuronal release of opioid peptides in the brain, then it would be expected that the biological activity of the peptide would be increased by peptidase inhibitors that are known to interfere with its degradation. There is much evidence to indicate that several peptidases are involved in the metabolism of endogenous opioid peptides, and a number of relatively selective inhibitors of these enzymes have been identified: endopeptidase 24.11 (EC 3.4.24.11) (18,25) is inhibited by phosphoramidon (14); aminopeptidase (EC 3.4.11.2) (7,10) is inhibited by bestatin (1,29); and angiotensin-coverting enzyme (ACE, EC 3.4.15.1) (9) is inhibited by captopril (6). Lower concentrations of these inhibitors were administered to attain a degree of enzyme inhibition without expression of an overt antinociceptive effect. In the

TABLE 1

INFLUENCE OF ANTISERA (AS) PRETREATMENT ON NITROUS OXIDE AD_{50} VALUES

Drug Pretreatment	Nitrous Oxide AD_{50} (95% Confidence Limits)
Control serum (4 μl, ICV)	34.2% (23.2–50.4%)
β-EP AS (100 μg, ICV)	34.3% (26.1–45.0%)
ME AS (100 μg, ICV)	36.1% (28.8–45.3%)
DYN 1–8 AS (100 μg, ICV)	56.6% (47.2–68.0%)*
DYN 1–13 AS (100 μg, ICV)	56.9% (50.0–64.7%)*

Significance of difference: *$p < 0.05$, compared to the control serum group.

TABLE 2

INFLUENCE OF PEPTIDASE-INHIBITOR PRETREATMENT ON NITROUS OXIDE AD_{50} VALUES

Drug Pretreatment	Nitrous Oxide AD_{50} (95% Confidence Limits)
Saline (4 μl, ICV)	42.5% (32.7–59.8%)
Phosphoramidon (3.0 μg, ICV)	25.0% (19.9–31.5%)*
Bestatin (10 μg, ICV)	40.4% (33.5–48.8%)
Captopril (300 ng, ICV)	38.0% (32.5–44.5%)

Significance of difference: *$p < 0.05$, compared to the saline control group.

case of phosphoramidon, for example, previous studies indicate that the active dose range for antinociception is 10–100 μg (19); therefore, a dose of 3.0 μg was utilized to increase the store of releasable opioid peptide in response to nitrous oxide.

In the present investigation, nitrous oxide antinociception was significantly potentiated by phosphoramidon but not bestatin and captopril. The results of these experiments with peptidase inhibitors are consistent with a report that DYN-induced antinociception in the mouse formalin test was potentiated by phosphoramidon as well as the cysteine proteinase inhibitor *p*-hydroxymercuribenzoate but not by bestatin, captopril, or the serine proteinase inhibitor phenylmethanesulfonyl fluoride (27).

It should also be mentioned that the other experimental paradigm that has been widely employed to study nitrous oxide antinociception is the rat hot-plate test. In this paradigm, nitrous oxide antinociception appears to involve the neuronal release of β-EP with subsequent activation of μ- and possibly ε-opioid receptors. This was deduced from attenuation of the nitrous oxide response by the μ-opioid receptor blocker CTP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2), as well as a rabbit antiserum against β-EP; on the other hand, the κ-opioid antagonist norbinaltorphimine and the δ-opioid antagonist naltrindole were ineffectual reducing nitrous oxide antinociception in the rat hot-plate test (12). The difference in mediating opioid peptides and opioid receptors may reflect a paradigm specificity rather than a species specificity. This is based on the generalization that nociceptive tests that utilize a thermal noxious stimulus (e.g., hot plate test, tail-flick test) can mobilize antinociceptive pathways involving μ- and δ- but not κ-opioid systems; on the other hand, those that employ a chemical noxious stimulus (e.g., abdominal constriction test) mobilize antinociceptive pathways mediated by μ- and κ- but not δ-opioid systems (28).

The results of the antisera experiments in the present investigation show that nitrous oxide antinociception was sensitive to antagonism by antisera against DYNs but not against β-EP or ME. The results of the peptidase inhibitor experiments are likewise consistent with the hypothesis that DYN is the causative peptide in nitrous oxide antinociception in the mouse abdominal constriction test.

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