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A Prolonged NO-Dependent, Opioid-Mediated Antinociceptive Effect of Hyperbaric Oxygen in Mice

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Abstract

Hyperbaric oxygen (HBO_2) therapy is reported to cause pain relief in several conditions of chronic pain. A single 60-min session of HBO_2 treatment produced a prolonged antinociceptive effect in mice that persisted for 90 min after cessation of treatment. The HBO_2-induced antinociception was significantly attenuated by pretreatment prior to HBO_2 exposure with the opioid antagonist naltrexone, the non-specific nitric oxide synthase (NOS)-inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) and the selective neuronal NOS-inhibitor S-methyl-L-thiocitrulline (SMTC) but not the selective endothelial NOS-inhibitor N^5-(1-iminoethyl)-L-ornithine (L-NIO). The antinociception was also significantly reduced by central pretreatment with a rabbit antiserum against dynorphin$_{1\text{-}13}$ but not by rabbit antisera against either β-endorphin or methionine-enkephalin. The prolonged antinociceptive effect at 90 min after HBO_2-induced treatment was also significantly attenuated by naltrexone but not L-NAME administered 60 min following HBO_2 treatment but prior to nociceptive testing. These findings indicate that the antinociception that persists for 90 min after HBO_2 exposure is mediated by nitric oxide (NO) and opioid mechanisms but that the NO involvement is critical during the HBO_2 treatment and not at the time of nociceptive testing. These results are consistent with the concept that HBO_2 may induce an NO-dependent release of opioid peptide to cause a long-acting antinociceptive effect.

Words for Indexing

Hyperbaric Oxygen; Nitric Oxide; Opioid; Antinociception; Mice

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Perspective

This article present evidence of a persistent antinociceptive effect of hyperbaric oxygen treatment that is mediated by opioid and nitric oxide mechanisms. Further elucidation of the underlying mechanism could potentially identify molecular targets to cause a longer-acting activation of endogenous pain-modulating systems.

Introduction

Hyperbaric oxygen (HBO_2) therapy is the clinical application of 100% oxygen at atmospheric pressures higher than sea level for limited periods of time (60–90 min) daily to achieve therapeutic outcomes. Normally at sea level or 1 atmosphere absolute (1 ATA), the partial pressure of oxygen (PaO_2) at the lungs breathing air (21% oxygen) is 102 mm Hg. By breathing 100% oxygen, the PaO_2 is elevated to 673 mm Hg at 1 ATA. But if the pressure is increased to 2 ATA, it is increased to 1433 mm Hg, a 14-fold increase in PaO_2 as compared to breathing air at 1 ATA, thus representing a significant increase in oxygen delivery to tissues[12].

The Committee on Hyperbaric Oxygen Therapy of The Undersea & Hyperbaric Medical Society (UHMS) evaluates clinical indications for HBO_2 treatment and makes recommendations in its Hyperbaric Oxygen Therapy Committee Report[9]. These recommendations have been approved by the U.S. Food and Drug Administration (FDA). The use of HBO_2 therapy for indications other than the thirteen UHMS-approved indications is considered off-label.

Among conditions that are reportedly responsive to HBO_2 are a variety of clinical conditions of pain for which exposure to HBO_2 causes a long-lasting analgesic effect. These painful conditions include complex regional pain syndrome[14,21,28], fibromyalgia syndrome[30], migraine and cluster headache[5,16,29], and pain associated with radiotherapy of cancer[4,13,17].

These reports suggest that HBO_2 treatment activates an endogenous pain-relieving mechanism whose continued activation does not require continued exposure to HBO_2 for persistence of pain relief. The purpose of the present study was to examine the duration of antinociceptive effect induced by a single 60-min HBO_2 treatment in comparison with another well-studied pharmacological gas, nitrous oxide (N_2O). Another aim was to determine whether this antinociceptive effect of HBO_2 is similar to that of N_2O in being mediated by opioid- and nitric oxide (NO) mechanisms.

Materials and Methods

Animals

Male NIH Swiss mice, weighing 18–22 g, were purchased from Harlan Laboratories (Indianapolis, IN) and used in this study, which 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).

Mice were housed five per cage in the AAALAC-accredited Wegner Hall Vivarium with access to food and water *ad libitum*. The facility was maintained on a 12-h light:dark cycle (lights on 0700–1900 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. All measures to minimize pain or discomfort were taken by the investigators.

Exposure to Hyperbaric Oxygen (HBO_2)

Cages of five mice each were placed in a B-11 research hyperbaric chamber (Reimers Systems, Inc., Lorton, VA) as previously described[20]. The chamber was ventilated with 100% O_2, U.S.P. (A-L Compressed Gases, Inc., Spokane, WA) at a flow rate of 20 L/min to minimize CO_2 accumulation. The pressure within the cylindrical clear acrylic chamber (27.9 cm diameter × 55.9 cm L) was increased at a rate of 1.0 ATA/min to the desired pressure and maintained for

60 min. The mice were allowed to breathe spontaneously during HBO_2 treatment. After completion of the HBO_2 exposure, mice were then decompressed at a rate of 1.0 ATA/min. Control groups of mice were exposed to compressed air (A-L Compressed Gases) circulated through the chamber at 1.0 ATA and maintained for 60 min. Decompression occurred as described above.

Exposure to Nitrous Oxide (N_2O)

N_2O, U.S.P. and O_2, U.S.P. (A-L Compressed Gases) 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% N_2O and 30% O_2 in the box was verified using a POET II® anesthetic monitoring system (Criticare, Milwaukee, WI). The total exposure time was 60 min. Exhausted gases were routed by polyethylene tubing to a nearby fume hood.

Antinociceptive Testing

Antinociceptive responsiveness was assessed as previously described[7], using the abdominal constriction test at various times following cessation of HBO_2 or N_2O treatment. 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® 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. 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 1300 and 1700 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:

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

Drugs

The following drugs were used in this research: naltrexone hydrochloride (NTX) (Tocris Bioscience, Ellisville, MO); N^G-nitro-L-arginine methyl ester (L-NAME) (Sigma Aldrich Research Biochemicals, Inc., St. Louis, MO); S-methyl-L-thiocitrulline (SMTC) (Sigma Aldrich); N^5-(1-iminoethyl)-L-ornithine (L-NIO) (Alexis Biochemicals Corporation, San Diego, CA); and rabbit antiserum against rat dynorphin $A_{1\text{-}13}$ (DYN AS), β-endorphin (β-EP AS) and methionine-enkephalin (ME AS) (Bachem/Peninsula Laboratories, LLC, San Carlos, CA).

NTX, L-NAME, SMTC and L-NIO were freshly prepared in 0.9% physiological saline solution. NTX and L-NIO were administered systemically (30-min pretreatment time) and L-NAME and SMTC were administered i.c.v. (15-min pretreatment time). In one set of experiments (#1, #2, and #3), opioid antagonists and NOS-inhibitors were administered 15–30 min prior to the 60-min HBO_2 treatment (180 min prior to antinociceptive testing). In another experiment (#4), opioid antagonist and NOS-inhibitor pretreatment was administered 60 min following cessation of the 60-min HBO_2 treatment (15–30 min prior to antinociceptive testing). For i.p. or s.c. pretreatments, the volume of injection was 0.1 ml/10 g body weight with control animals receiving an i.p. or s.c. injection of vehicle (sterile saline) only. For i.c.v. pretreatments, the volume of microinjection was 5.0 μl per mouse with control animals receiving an i.c.v. microinjection of vehicle (sterile saline) only.

DYN AS, β-EP AS and ME AS lyophilates were reconstituted in distilled water; the final antiserum solutions contained 0.1-M phosphate buffered saline (PBS, pH 7.4). The antisera were microinjected in an i.c.v. dose of 10 μg 30 min prior to the 60-min HBO_2 treatment (180 min prior to antinociceptive testing). The volume of i.c.v. microinjection was 5.0 μl per mouse. Control animals received an i.c.v. microinjection of vehicle (sterile saline or PBS) only.

Intracerebroventricular (i.c.v.) Microinjection Procedure

Intracerebroventricular (i.c.v.) pretreatments were made using a modification of a published microinjection technique[10]. Briefly, 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-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.

Statistical Analysis of Data

A two-way analysis of variance (2 ANOVA) and a one-way ANOVA (1 ANOVA) with a *post-hoc* Bonferroni multiple comparison test was used to analyze the time-dependent antinociceptive effects of HBO_2 and N_2O. Percent changes in antinociception were arcsine-transformed prior to statistical analysis. A one-way ANOVA with a *post-hoc* Bonferroni multiple comparison test was used to compare HBO_2-induced antinociception various pretreatment groups.

Results

In Experiment #1, mice were subjected to 60 min of HBO_2 or N_2O treatment then removed to room air. Different groups of mice were injected with 0.6% glacial acetic acid and assessed for antinociception at 5, 15, 30, 60, 90, 120, 150 or 180 min following exposure. The 0-time groups were removed to room air following 60 min of HBO_2 or N_2O treatment and were immediately injected with glacial acetic acid for antinociceptive testing 5 min later [2 ANOVA between 0–90 min, Treatment; $F_{1,101} = 114.4$, $p < 0.0001$, Time; $F_{5,101} = 18.8$, $p < 0.0001$, Interaction; $F_{5,101} = 10.45$, $p < 0.0001$]. HBO_2 treatment produced responses that were 80–95% of the maximal antinociceptive effect through 90 min (Fig. 1). At 120 min, the antinociceptive effect fell to 40% of maximum and further declined to under 20% by 180 min after HBO_2 treatment [1 ANOVA; $F = 20.25$, $p < 0.0001$]. By comparison, N_2O treatment caused mice to exhibit at least 70% antinociception at 5 and 15 min after exposure, but the response declined to approximately 20% by 30 through 90 min [1 ANOVA; $F = 25.24$, $p < 0.0001$].

In Experiment #2, different groups of mice were pretreated with NTX (3.0 mg/kg, i.p.), L-NAME (1.0 μg/mouse, i.c.v.), SMTC (1.0 μg/mouse, i.c.v.) or L-NIO (3.0 mg/kg, s.c.) 15–30 min prior to HBO_2 treatment. The antinociceptive effect assessed 90 min after HBO_2 treatment was completely abolished by NTX and L-NAME, antagonized by two-thirds by SMTC and largely unaffected by L-NIO [1 ANOVA; $F = 25.57$, $p < 0.0001$] (Fig. 2). In preliminary experiments, different groups of animals were treated with NTX, L-NAME, SMTC or L-NIO in the absence of HBO2 treatment; none of these pretreatments alone (at the doses used) had any appreciable effect on the number of acetic acid-induced abdominal constrictions (data not shown).

In Experiment #3, different groups of mice were pretreated with DYN AS (10 μg/mouse, i.c.v.), β-EP AS (10 μg/mouse, i.c.v.), and ME AS (10 μg/mouse, i.c.v.) or a cocktail of DYN AS (10 μg/mouse, i.c.v.) and β-EP AS (10 μg/mouse, i.c.v.) 30 min prior to HBO_2 treatment [1 ANOVA; $F = 27.49$, $p < 0.0001$]. DYN AS was the most effective antagonist of the antinociception assessed 90 min following cessation of HBO_2 treatment, reducing the magnitude of the response by one-half (Fig. 3). The β-EP antiserum reduced HBO_2-induced antinociception by about one-fifth, and the ME antiserum did not appreciably influence the antinociceptive effect. The combination of DYN AS and β-EP AS reduced HBO_2-induced antinociception by two-thirds, but this was not statistically different from the response of mice pretreated with DYN AS. In preliminary experiments, different groups of animals were treated with DYN AS, β-EP AS or ME AS in the absence of HBO_2 treatment; none of these antiserum pretreatments alone (at the doses used) had any appreciable effect on the number of acetic acid-induced abdominal constrictions (data not shown).

In Experiment #4, different groups of mice were pretreated with either NTX (3.0 mg/kg, i.p.) or L-NAME (1.0 μg/mouse, i.c.v.) 60 min *after* HBO_2 treatment and 30 min prior to antinociceptive testing [$F = 58.04$, $p < 0.0001$]. NTX significantly antagonized the magnitude of the HBO_2-induced antinociception assessed 90 min after HBO_2 treatment, while the L-NAME pretreatment had no effect at all (Fig. 4).

Discussion

An involvement of NO in mediating the effects of HBO_2 treatment is in agreement with previous studies. In *in vivo* brain microdialysis experiments conducted in rats, HBO_2 treatment at 3 ATA for 120 min increased hippocampal and striatal levels of NO metabolites; both these increases in dialysate levels of NO metabolites were blocked by pretreatment with L-NAME[6]. In another study, NO-specific and O_2-specific electrodes were implanted in the brains of anesthetized rats subsequently exposed to HBO_2 at 2.0–2.8 ATA for ~20 min; the increase in tissue NO levels increased in proportion to increases in the tissue O_2 concentration and was sensitive to antagonism by 7-nitroindazole, a neuronal-selective NOS-inhibitor[27].

Our laboratory also recently demonstrated that HBO_2-specific NO changes increases in different regions of the rat brain and spinal cord[20]. Exposure to 100% O_2 alone (NBO_2) generally decreased regional brain and spinal cord levels of the NO metabolites, nitrite and nitrate, while exposure to compressed air at 2.5 ATA (NBA) had little effect on tissue levels of NO metabolites. However, the combination of 100% oxygen and pressure (*i.e.*, HBO_2) generally increased tissue levels of nitrite and nitrate, which is indicative of increased NO in selected brain regions, most notably in the corpus striatum, brainstem, cerebellum and spinal cord.

HBO_2 is approved by the FDA for certain clinical indications[9], although there is anecdotal evidence and clinical reports of HBO_2 efficacy in a broader range of conditions. Among conditions that are reportedly responsive to HBO_2 are a variety of clinical conditions of pain. Patients suffering from complex regional pain syndrome (CRPS)—formerly called reflex sympathetic dystrophy (RSD)—experienced less pain following HBO_2 therapy[14,21,28]. Significant pain reduction was also reported in patients with generalized allodynia/hyperalgesia as a consequence of fibromyalgia syndrome (FMS) [30]. Patients suffering from migraine headache or cluster headache also reportedly experienced pain relief following HBO_2 therapy[5,16,29]. Pain associated with radiotherapy of cancer has also been reported to be alleviated by HBO_2 treatment4,13,17.

A number of these clinical studies indicate pain relief for varying periods of time following one or more sessions of HBO_2 therapy. This suggests that HBO_2 treatment activates an

endogenous pain-relieving mechanism whose continued activation does not require continued exposure to HBO_2 for persistence of pain relief. To determine whether HBO_2 treatment (2.5 ATA for 60 min) in our animals activated pain-relieving mechanisms that outlast the period of its actual exposure sojourn, different groups of mice were tested for antinociceptive responsiveness at varying time intervals after cessation of HBO_2 treatment.

The results demonstrate clearly that both HBO_2 and N_2O treatments can reduce the number of acetic acid-induced abdominal constrictions in mice as we have previously demonstrated[31]. But, the N_2O-induced antinociception starts to dissipate after 15 min following termination of 60-min exposure to N_2O, the HBO_2-induced antinociception lasts at least 90 min following a single 60-min session of HBO_2 treatment. N_2O is not metabolized by the body and, due to its low blood/gas partition coefficient, is largely eliminated in the expired air[25]. The antinociceptive effect that lasts for 15 min following termination of exposure may reflect the residual pharmacological activity of the neuronally-released endogenous opioid peptide(s) which is limited by enzymatic degradation.

By comparison, the duration of the post-HBO_2 antinociception is six times longer than that of N_2O. During and following the HBO_2 treatment (2.5 ATA, 100% O_2), tissue O_2 concentrations would be higher, resulting in significantly increased biosynthesis of NO[26]. The increased tissue NO levels may lead to a greater and more prolonged release of endogenous opioid peptide as compared to the N_2O exposure at 1 ATA (30% O_2). Another possible explanation is that HBO_2 exposure activates an endogenous antinociceptive pathway that is longer acting than the pathway activated by N_2O.

This HBO_2-induced antinociceptive effect was nearly completely antagonized by systemic pretreatment with NTX, an opioid receptor blocker, and by an i.c.v. administration of the non-selective NOS-inhibitor, L-NAME. L-NIO, selective endothelial NOS-inhibitor, produced a slight (10%) reduction in HBO_2-induced antinociception which was not statistically significant. However, following pretreatment with SMTC (i.c.v.), a selective inhibitor of neuronal NOS, the HBO_2-induced antinociception was significantly antagonized. These results indicate that the antinociceptive pathway involves the O_2-induced biosynthesis of NO via neuronal NOS and implicates the opiate receptors. This agrees with our earlier findings with respect to N_2O-induced antinociception[11,15,24]. The antinociceptive response was likewise antagonized by NTX, L-NAME and SMTC. We have previously hypothesized that N_2O-induced antinociception results from a stimulated NO-dependent neuronal release of endogenous opioid peptides[8].

The antinociceptive effect produced by HBO_2 treatment was significantly antagonized by pretreatment with DYN AS, the magnitude of the antinociceptive response being reduced by roughly one-half. Neither β-EP AS or ME AS produced a significant reduction in the HBO_2-induced response. When mice were pretreated with a cocktail of DYN AS and β-EP AS, the HBO_2-induced antinociceptive effect was reduced by two-thirds; however, this was not significantly different from the DYN AS-antagonized response to HBO_2. It would appear that DYN—an opioid peptide with known antinociceptive properties[17,22]—is the primary mediator of HBO_2-induced antinociception. Once again, this is similar to observations made in studies of N_2O-induced antinociception. Supraspinal pretreatment of mice with DYN AS significantly antagonized the antinociceptive of N_2O, while β-EP AS and ME AS were without effect[1,2].

In other studies, i.c.v. administration of the NO precursor L-arginine (L-ARG) or the NO-donor 3-morpholinosydnoimine (SIN-1) evoked antinociceptive effects in the mouse abdominal constriction test[3]. These antinociceptive responses were sensitive to antagonism by naloxone and DYN AS, suggesting the antinociceptive response resulted from DYN activating opioid

receptors in the brain. The functional link between NO and release of DYN was provided by the finding that pretreatment with the neuronal-selective NOS-inhibitor SMTC blocked the antinociceptive response to L-ARG but not SIN-1. This is reinforced by results from microdialysis experiments conducted in rats, in which N_2O-induced increases in levels of both β-EP and NO metabolites in samples collected from the arcuate nucleus were antagonized by pretreatment with L-NAME[19].

These findings are consistent with the hypothesis that both N_2O and HBO_2 may produce antinociception through stimulation of an NO-dependent neuronal release of the opioid peptide DYN[8]. If NO does, indeed, play a regulatory role in the neuronal release of opioid peptides, this might explain why NOS-inhibitors can antagonize HBO_2-induced antinociception during the actual HBO_2 treatment rather than at the time of nociceptive testing. Once endogenous opioid peptides have been released from their neuronal sources, NO is no longer required for expression of the antinociceptive response.

This research will potentially identify molecular targets that can cause prolonged activation of endogenous pain-relieving systems. It remains to be seen whether multiple sessions of HBO_2 treatment might produce an antinociceptive response of even longer duration. Although HBO_2 therapy is not at present indicated in the treatment of pain *per se*, it may represent a new weapon in the pharmacological armamentarium for clinical management of pain.

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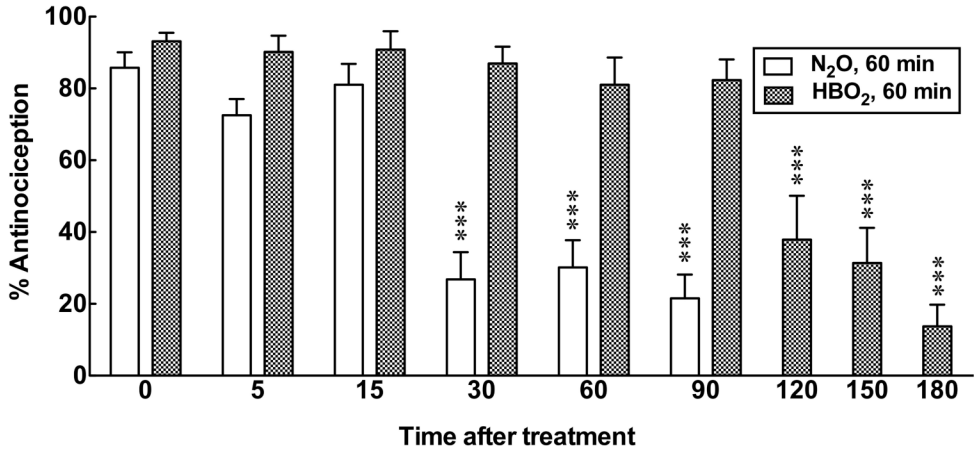

Fig. 1.

A comparison of the duration of HBO_2- versus N_2O-induced antinociceptive effects. Each bar represents the mean percent of antinociceptive response ± S.E.M. of 8–12 mice per group. Significance of difference: ***, $p < 0.001$, compared to the 0 time N_2O or HBO_2 control group (post-hoc Bonferroni test).

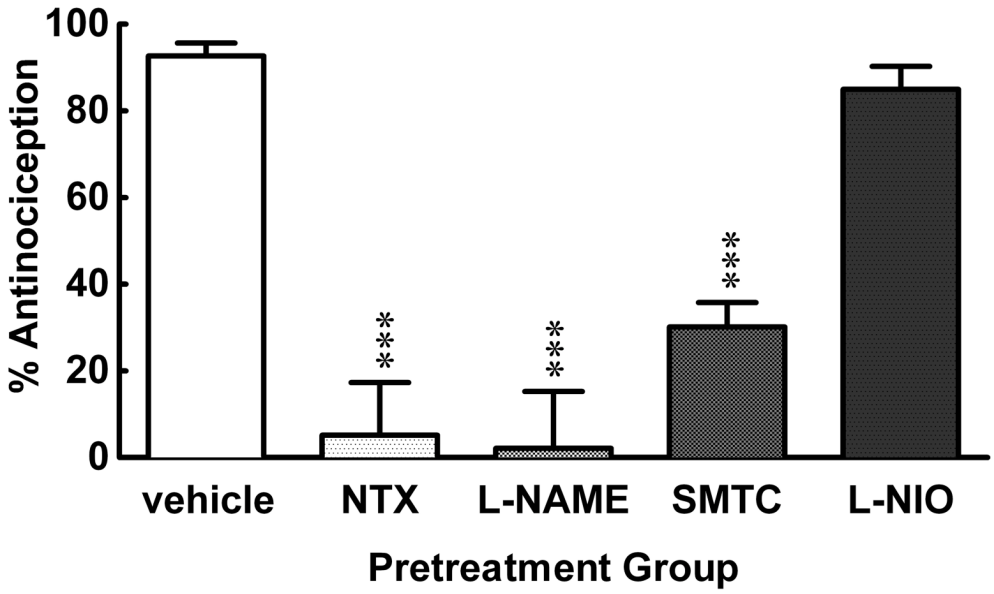

Fig. 2.

Influence of opioid antagonist and NOS-inhibitor pretreatments administered prior to HBO_2 treatment in mice. The data are expressed as the mean ± S.E.M. of 8–12 mice per group. Significance of difference: ***, $p < 0.001$, compared to the vehicle pretreatment group (post-hoc Bonferroni test).

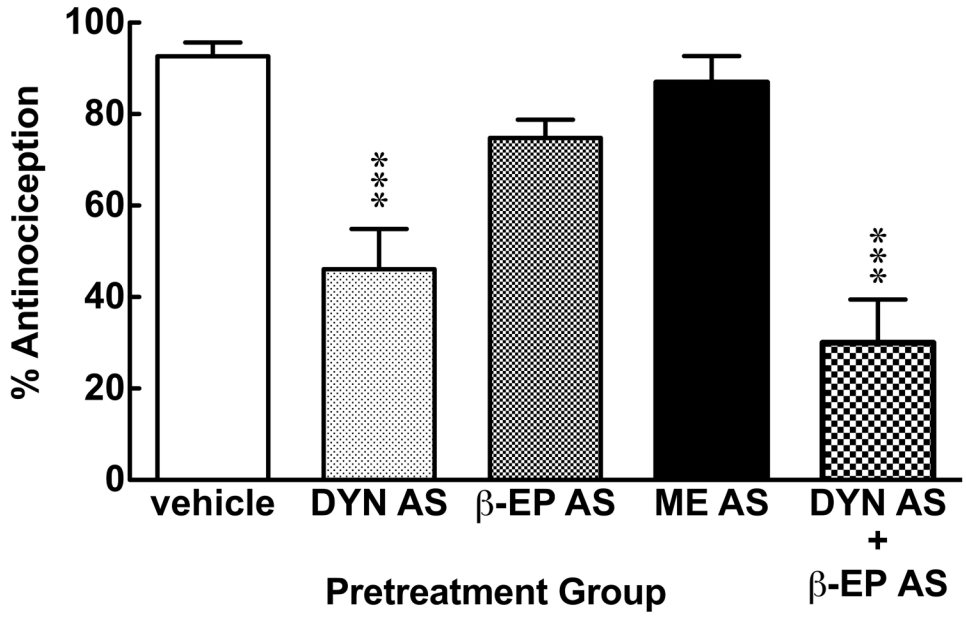

Fig. 3.

Influence of opioid peptide antiserum pretreatment administered before HBO_2 treatment in mice. The data are expressed as the mean ± S.E.M. of 6–12 mice per group. Significance of difference: ***, $p < 0.001$, compared to the vehicle pretreatment group (post-hoc Bonferroni test).

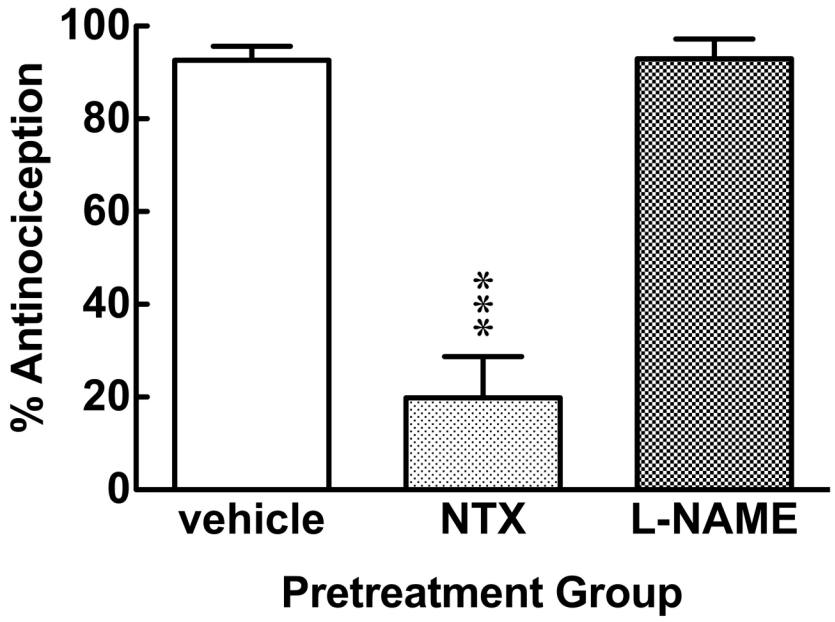

Fig. 4.

Influence of opioid antagonist and NOS-inhibitor pretreatment administered after HBO_2 treatment and prior to antinociceptive testing in mice. The data are expressed as the mean ± S.E.M. of 8–12 mice per group. Significance of difference: ***, $p < 0.001$, compared to the vehicle pretreatment group (post-hoc Bonferroni test).