Hyperbaric Oxygen Treatment Induces a 2-Phase Antinociceptive Response of Unusually Long Duration in Mice

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Abstract: Hyperbaric oxygen (HBO₂) therapy is approved by the FDA for limited clinical indications but is reported to produce pain relief in several chronic pain conditions. However, there have been no studies to explain this apparent analgesic effect of HBO₂. Research conducted in our laboratory demonstrates that 4 daily 60-minute HBO₂ treatments at 3.5 absolute atmospheres induced an unparalleled antinociceptive response that consists of 1) an early phase that lasted at least 6 hours after the HBO₂ treatment before dissipating; and 2) a late phase that emerged about 18 hours after the early phase and lasted for up to 3 weeks. The early phase was sensitive to antagonism by acutely intracerebroventricular (i.c.v.)-administered opioid antagonist naltrexone and the nitric oxide synthase (NOS)-inhibitor L-NAME. The late phase was inhibited by treatment with i.c.v. naltrexone or L-NAME during the 4 daily HBO₂ treatments but was not antagonized by either naltrexone or L-NAME following acute pretreatment 2 weeks after HBO₂ treatment. These experimental results implicate a novel mechanism that is activated by HBO₂, resulting in an antinociceptive response of unusually long duration that is of potential interest in the clinical management of pain.

Perspective: Hyperbaric oxygen treatment of mice can induce a 2-phase antinociceptive response of unusually long duration. Nitric oxide and opioid receptors appear to initiate or mediate both phases of the antinociceptive response. Further elucidation of the underlying mechanism may potentially identify molecular targets that cause long-lasting activation of endogenous analgesic systems.

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reduced paw swelling) as well as mechanical hypersensitivity (as determined by increased threshold for paw withdrawal). The ability of HBO2 treatment to reduce inflammation and pain was comparable to that of acetylsalicylic acid treatment. It is notable that there was a temporal dissociation of the antinflammatory and antinociceptive effects. The anti-inflammatory effect was almost immediate after carrageenan was administered to HBO2-treated rats, while the antinociceptive effect did not manifest itself until nearly 2 hours after the HBO2 treatment.

We recently reported that a 60-minute exposure to 100% oxygen (O2) at 3.5 absolute atmospheres produced an antinociceptive effect of at least 90 minutes' duration that involves opioid and nitric oxide (NO) mechanisms. Mice exposed to HBO2 for 60 minutes were returned to room air, after which antinociception was assessed. The antinociceptive effect was significantly attenuated by intracerebroventricular (i.c.v.) pretreatment with 2 inhibitors of nitric oxide synthase (NOS) enzyme, the nonselective inhibitor \( N^\bullet \)-nitro-L-arginine methyl ester (L-NAME), and the neuronally-selective inhibitor \( S^- \)methyl-L-thiocitrulline (SMTC). The endothelial-selective NOS-inhibitor \( N^\bullet \)-((1-iminoethyl)-L-ornithine (L-NIO) administered ip just prior to the start of HBO2 treatment had no effect on the antinociceptive response. The antinociceptive effect at 90 minutes was also markedly antagonized by ip pretreatment with the opioid receptor blocker naltrexone. Confirming an involvement of endogenous opioid peptides in the antinociception, the effect was found to be sensitive to antagonism by i.c.v. pretreatment with a rabbit antiserum against rat dynorphin but not by antisera against either \( \beta^- \)endorphin or methionine-enkephalin. The prolonged antinociceptive effect at 90 minutes after HBO2-induced treatment was also significantly attenuated by naltrexone—but not L-NAME—administered 75 minutes following HBO2 treatment but 15 minutes prior to nociceptive testing. Based on these experimental findings, we concluded that the HBO2-induced antinociceptive effect involves both NO and opioid mechanisms in the brain and is consistent with our hypothesis that HBO2 can stimulate an NO-dependent neuronal release of dynorphin, which, in turn, activates \( \kappa \) opioid receptors that mediate antinociception.

The present study grew out of an attempt to determine whether repeated sessions of HBO2 treatment might prolong the duration of the HBO2-induced antinociception and to pharmacologically characterize the antinociceptive response.

**Methods**

**Animals**

Male NIH Swiss mice, weighing 18 to 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). All measures to minimize pain or discomfort were taken by the investigators.

Mice were housed in the AAALAC-accredited Wegner Hall Vivarium with access to food and water ad libitum. The facility was maintained on a 12-hour light/dark cycle (lights on 0700 to 1900 hours) under standard conditions (22 ± 1°C room temperature, 33% humidity). Mice were kept in the holding room for at least 4 days after arrival in the facility for acclimation prior to experimentation.

**Exposure to Hyperbaric Oxygen (HBO2)**

Cages of 5 mice each were placed in a B-11 research hyperbaric chamber (Reimer’s Systems, Inc.; Lorton, VA) as previously described. The chamber was ventilated with 100% oxygen (O2), U.S.P. (A-L Compressed Gases, Inc, Spokane, WA) at a flow rate of 20 L/minute to minimize carbon dioxide accumulation. The pressure within the cylindrical clear acrylic chamber (27.9 cm diameter \( \times \) 55.9 cm L) was increased at a rate of 1.0 ATA/min to the desired pressure (3.5 ATA) and maintained for 60 minutes. The mice were allowed to breathe spontaneously during HBO2 treatment. After completion of the HBO2 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 minutes. Decompression typically took 4-5 min.

Mice were subjected to HBO2 treatment at 3.5 ATA for 60 minutes between 1000 and 1100 hours for 4 consecutive days. At different time intervals following the fourth HBO2 session, different groups of mice were assessed for antinociceptive responsiveness, as described in the following section, due to loss of sensitivity to acetic acid for several days following injection.

**Antinociceptive Testing**

Antinociceptive responsiveness was assessed using the abdominal constriction test as previously described. At varying time intervals following HBO2 treatment, different groups of mice were treated ip with .1 mL per 10 g body weight of .6% glacial acetic acid and placed into an open clear Plexiglas chamber (35 cm L \( \times \) 20 cm W \( \times \) 15 cm H). Exactly 5 minutes later, the number of abdominal constrications—lengthwise stretches of the torso with concave arching of the back—in each animal was counted for six-minute periods for each treatment group. Multiple raters were used for some but not all experiments; at least 1 of the raters was blinded to the drug treatment. The control reference group was exposed to room air. The degree of antinociception (inhibition of abdominal constrications) produced in various treatment groups of mice was calculated as:

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

A mean number of abdominal constrications was determined for control mice and was used as the basis for
determining the percent antinociceptive response for each experimental mouse at a specific time point or in a particular pretreatment group. In the time-course experiment, different groups of at least 6 mice were tested at each time interval following HBO2 treatment because the ip injection desensitizes for up to 5 days the sensory receptors that initiate the abdominal constrictions.

Drugs

The following drugs were used in this research: naltrexone hydrochloride (Tocris Bioscience, Ellisville, MO); and L-NG-nitro arginine methyl ester (L-NAME) (Research Biochemicals International, Natick, MA). Naltrexone and L-NAME were freshly prepared daily in sterile 9% physiological saline solution and administered acutely or chronically into the lateral cerebral ventricle. Control animals received vehicle (physiological saline solution) via the same route.

Acute Intracerebroventricular Microinjection Procedure

Acute i.c.v. microinjections of 1.0 μg naltrexone or L-NAME were made using the microinjection technique of Haley and McCormick. Briefly, mice were anesthetized with isoflurane, U.S.P. (Abbott Laboratories, N. 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; the hypodermic needle which served as a collar to limit penetration of the microsyringe needle to 2.0 mm. A volume of 4.0 μL of drug solution was delivered directly into the lateral cerebral ventricle over 30 seconds.

Chronic Intracerebroventricular Infusion Procedure

For chronic i.c.v. delivery of 1.0 μg/day naltrexone and L-NAME during the 4-day HBO2 treatment, mice were anaesthetized with isoflurane and brain infusion cannulae (Alza, Cupertino, CA) were implanted into the lateral cerebral ventricle. Each brain infusion cannula was connected to a dorsally, subcutaneously implanted Alzet osmotic minipump model 2001 (200-μL reservoir, 1.0 ± .04 μL/hr pumping rate, delivers for up to 1 week) with polyvinylchloride tubing. The concentration of naltrexone and L-NAME stored in each minipump was .0416 μg/μL. The osmotic minipumps were implanted prior to the first HBO2 treatment and were removed immediately following the fourth HBO2 treatment.

Statistical Analysis of Data

Percent changes in antinociception were arc-sine-transformed to normalize the distribution of percentages prior to statistical analysis. A 1-way ANOVA and post hoc Bonferroni’s multiple comparison test was used to compare HBO2-induced antinociception in various pretreatment groups.

Results

Time Course of HBO2 -Induced Antinociception

Fig 1 shows the time course of the biphasic antinociceptive response following 4 daily HBO2 treatments at 3.5 ATA of 60 minutes each. HBO2 treatment produced a robust antinociceptive response (approximately 90 to 95% suppression of abdominal constrictions) that lasted for up to 6 hours following the last HBO2 session.

When antinociceptive testing was conducted after additional time intervals, it was discovered that the antinociceptive effect of HBO2 had completely dissipated by
12 hours but began to re-emerge 24 hours after the last HBO2 treatment. This delayed antinociceptive effect—now called the late-phase response to differentiate it from the 6-hour-long early-phase effect—proved to be equal in antinociceptive intensity to the earlier response and persisted for 14 days after the last HBO2 session. At 21 days after the last session, the antinociceptive response was still at 40%.

**Influence of Acute Naltrexone and L-NAME Pretreatment on the HBO2-Induced Early-Phase Antinociceptive Response**

Fig 2 shows the influence of naltrexone and L-NAME pretreatments 3 hours after the last HBO2 treatment and 30 minutes prior to the glacial acetic acid challenge. I.c.v. pretreatment with 1.0 μg naltrexone caused a 50% reduction in the magnitude of the HBO2-induced antinociceptive effect. Similar i.c.v. pretreatment with 1.0 μg L-NAME reduced the HBO2-induced response by 40%.

**Influence of Acute Naltrexone and L-NAME Pretreatment on the HBO2-Induced Late-Phase Antinociceptive Response**

We then assessed the influence of naltrexone and L-NAME on the late-phase antinociceptive response following the 4-day HBO2 treatment. Fig 3 shows the influence of i.c.v.-administered naltrexone (1.0 μg) and L-NAME (1.0 μg) 14 days after the last HBO2 treatment and 30 minutes prior to the glacial acetic acid challenge. Neither pretreatment had any effect on the magnitude of the HBO2-induced late-phase antinociceptive effect.

**Influence of Continuous Naltrexone and L-NAME Pretreatment on the HBO2-Induced Late-Phase Antinociceptive Response**

Finally, we wanted to determine whether opioid receptor blockade or inhibition of NO production during HBO2 exposure had any effect on development of the late-phase antinociception. Naltrexone (1.0 μg/day) and L-NAME (1.0 μg/day) were continuously delivered into the lateral cerebral ventricle using osmotic minipumps during the 4-day period in which mice were exposed to HBO2 for 60 minutes each day. Immediately following the fourth day of HBO2 treatment, the osmotic minipumps were removed and mice were returned to the vivarium. Two weeks later, the mice were tested for nociceptive responsiveness to the glacial acetic acid. Fig 4 shows that both naltrexone and L-NAME treatments during HBO2 exposure significantly reduced the intensity of the HBO2-induced late-phase antinociceptive response.

**Discussion**

**HBO2 and Nitric Oxide Function**

During HBO2 therapy in humans breathing room air (21% O2) at 1.0 ATA, the alveolar pO2 (pAO2) is
relationship between pO2 and tissue NO concentration. So there appears to be a clear
641 nM NO concentration increased from 36 nM at 1.0 ATA to
the hippocampus and striatum. 7 In
48 metabolites (NOx)—nitrite (NO2−) and nitrate (NO3−)—in rat
anesthesia and spinal cord. Thus, these data suggest that at
hyperbaric pressures, molecular oxygen can be converted to NO.

There is always the potential that the effects observed in this study may be associated to O2 toxicity. Rats exposed to 5 to 6 ATA HBO2 have been shown to exhibit EEG seizure patterns.1,2,5 The onset of these seizures were delayed by the nNOS inhibitor, 7-nitroindazole, suggesting the involvement of NO and/or oxygen radicals. However, exposure for 75 minutes at 4 ATA did not demonstrate any seizure patterns.5 What these data suggest that, under our experimental condition of 3.5 ATA, our results do not appear to reflect a toxic effect of HBO2.

Recent research has also shown that mice can tolerate as much as 6.0 ATA HBO2 and exhibit seizures only if there is preconditioning by 60-minute twice daily HBO2 treatments at 2.5 ATA for 3 consecutive days.16 Preconditioning led to an increase in levels of protein and mRNA of eNOS and nNOS in the hippocampus and hypothalamus. Our 4 daily 60-minute HBO2 treatments in the present study did not appear to sensitize the mice to oxygen-induced toxicity.

Antinociceptive Responsiveness of Rats to HBO2 Treatment

All of the discussion above supports our recent report that antinociception occurs very rapidly, that is, within 5 minutes of HBO2 exposure at 3.5 ATA.20,34 If the HBO2 exposure is continued for 60 minutes, the antinociception is extended beyond the duration of the HBO2 exposure for at least 90 minutes and, at 150 minutes, about 40% of the antinociception still remained.33 The exact mechanism by which this HBO2-induced antinociception is prolonged is not clear at this time.

Interestingly, in the present study, administration of the 60-minute HBO2 treatment for 4 consecutive days extends the duration of antinociception during the early-phase response 4-fold to 6 hours. It is interesting to note here that the duration of this antinociceptive effect (early-phase antinociception) is a multiple of the 90-minute effect observed after a single HBO2 exposure. This may suggest that HBO2 treatment induces some type of up-regulation mechanism, which has been reported recently by others.3,5,15,23

Even more interesting is that after 4 daily 60-minute exposures of HBO2, a second antinociception period (ie, the late-phase response) is initiated 24 hours after the last HBO2 exposure. The level of antinociception increased over 4 days and peaked on the fifth day after the last HBO2 session. This peak level of antinociception
equaled that of the early-phase response in magnitude and was maintained for another 9 days, at which time it gradually decreased to about 45% antinociception by 3 weeks after HBO2 treatment. What this suggests is that an antinociceptive pathway is clearly being up-regulated in the absence of HBO2 exposure. That would suggest that there was activation and/or inhibition of particular genes in the central nervous system, meaning that changes in gene regulation had possibly occurred under HBO2 treatment.

Recently it was reported that rats treated daily for 5 days with HBO2 for 60 minutes at 3.5 ATA showed distinct changes in gene function in the hippocampal CA1 region as assessed by DNA microarray analysis. These HBO2-treated animals were subjected to forebrain ischemia. Ischemic neuronal damage in the hippocampal CA1 was determined and showed that the prior HBO2 treatment decreased the amount of neuronal damage. Seven genes with their respective proteins presumed to be related to the neuroprotective effect of HBO2 were found to be up-regulated. The peak gene expression occurred generally at 12 hours, and the Western blot analysis of their respective proteins peaked at 24 hours. If gene regulation is involved in the late-phase antinociceptive response in the present study, then it would appear that the half-life of the proteins involved in the antinociception observed is much longer than in the previous study. This requires a closer examination of the genes and proteins that might possibly be involved in the antinociceptive response.

**Characterization of the HBO2-Induced Early-Phase Antinociceptive Response**

The present results show that, after 4 daily 60-minute HBO2 treatments, the early-phase antinociceptive effect is sensitive to antagonism by naltrexone administered i.c.v. 3 hours after the HBO2 treatment. This suggests that the early-phase antinociceptive response was mediated by opioid receptors and is in agreement with our previous finding that naltrexone antagonized the antinociceptive effect demonstrated after a single 60-minute exposure to HBO2. It is highly unlikely that O2 directly interacts with opioid receptors, which are not known to contain a heme group in their molecular structure. In addition, there is no evidence that hyperoxemia leads to direct activation of opioid receptors. The early-phase antinociception was also antagonized by L-NAME, a NOS-inhibitor. This also agrees with our data on antagonism by L-NAME of the 90-minute antinociceptive response to a single 60-minute exposure to HBO2. The naltrexone and L-NAME antagonisms of the early-phase antinociceptive response might imply that there might be a relationship between NO and endogenous opiate release, as previously proposed.

**Characterization of the HBO2-Induced Late-Phase Antinociceptive Response**

Acute i.c.v. treatment with naltrexone 2 weeks after the 4-day HBO2 treatment had no effect on the late-phase antinociceptive response as contrasted with its influence on the early-phase antinociception. Similarly, acute i.c.v. administration of L-NAME failed to have any influence on the late-phase antinociceptive effect. This appears to indicate that the early- and late-phase antinociceptive effects of HBO2 are not immediately mediated by the same mechanisms.

By using osmotic minipump technology, L-NAME and naltrexone were continuously administered i.c.v. during the 4-day HBO2 treatment to determine their influence on HBO2-induced antinociception. The late-phase antinociception induced by HBO2 was inhibited ~80% by naltrexone and ~70% by L-NAME. Thus, it appears that there is an action of HBO2 at the time of exposure that requires NO and/or opioid receptor activation in order for the late-phase antinociceptive response to develop. But the final step in the antinociceptive pathway is downstream and is not immediately mediated by NO or opioid mechanisms.

The late-phase antinociceptive effect developed only after 4 daily 60-minute HBO2 treatments and was not evident after a single 60-minute HBO2 exposure. Multiple-dosing or exposures of patients or experimental animals to HBO2 appear to be very complicated. In the first instance, the usual HBO2 treatment is 60 to 90 minutes once a day, so the HBO2 exposure is relatively short over a 24-hour time period. This would necessarily imply that some endogenous mechanism is activated or involved to sustain the pharmacological effect of HBO2 beyond a 24-hour period. Up-regulation might be at least part of the answer.

A varying multiple-dosing schedule for HBO2 therapy has been reported in the treatment of patients with retinitis pigmentosa (RP). In this study, the patients were exposed to HBO2 at 2.2 ATA for 90 minutes. The exposure or dosing schedule was the following: daily exposure for 5 days for 4 weeks followed by 5 exposures once a month for 11 months and, finally, 5 exposures once every 3 months for 2 years. The results showed a significant increase in electroretinogram measurements as compared to controls. A 10-year study conducted by the same investigators employed the same dosing schedule but increasing the duration of the final regimen (5 exposures every 3 months) from 3 years to 10 years. Results showed that different visual acuity measurements were significantly higher than for controls. In these studies, the turnover of the functional proteins involved appeared to be relatively long, ie, approximately 3 months in duration. By comparison, in the present investigation, the turnover of the key proteins involved in the late-phase antinociceptive pathway appears to be somewhat shorter, about 2 to 3 weeks.

It remains to be determined whether increasing the number of HBO2 exposures or that periodic re-exposure to HBO2 will further extend the duration of the late-phase antinociceptive response analogous to the HBO2 treatment of RP cited above. This will be important to understand pharmacologically, since HBO2 may potentially be effective in treatment of different types of chronic pain.
References


