HHS Public Access

Author manuscript

Brain Res. Author manuscript; available in PMC 2019 November 15.

Published in final edited form as:

Brain Res. 2018 November 15; 1699: 107–116. doi:10.1016/j.brainres.2018.08.002.

Role of spinal GABA receptors in the acute antinociceptive response of mice to hyperbaric oxygen

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Abstract

New pain treatments are in demand due to the pervasive nature of pain conditions. Hyperbaric oxygen (HBO_2) has shown potential in treating pain in both clinical and preclinical settings, although the mechanism of this effect is still unknown. The aim of this study was to investigate whether the major inhibitory neurotransmitter γ-aminobutyric acid (GABA) is involved in HBO_2-induced antinociception in the central nervous system. To accomplish this goal, pharmacological interactions between GABA drugs and HBO_2 were investigated using the behavioral acetic acid abdominal constriction test. Western blotting was used to quantify protein changes that might occur as a result of the interactions. $GABA_A$ but not $GABA_B$ receptor antagonists dose-dependently reduced HBO_2 antinociception, while antagonism of the GABA reuptake transporter enhanced this effect. Western blot results showed an interaction between the pain stimulus and HBO_2 on expression of the phosphorylated β3 subunit of the $GABA_A$ receptor at S408/409 in homogenates of the lumbar but not thoracic spinal cord. A significant interaction was also found in neuronal nitric oxide synthase (nNOS) expression in the lumbar but not thoracic spinal cord. These findings support the notion that GABA may be involved in HBO_2-induced antinociception at the $GABA_A$ receptor but indicate that more study will be needed to understand the intricacies of this interaction.

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Keywords

Hyperbaric oxygen; GABA receptors; Nitric oxide synthase; Spinal cord; Antinociception

1. Introduction

HBO_2 therapy is the use of 100% oxygen (O_2) at higher-than-atmospheric pressures for limited periods of time (generally 60–90 min) to achieve beneficial clinical outcomes. HBO_2 therapy is approved by the U.S. Food and Drug Administration (FDA) for only limited clinical indications, which include decompression sickness, carbon monoxide poisoning, wound healing and delayed radiation injury (Weaver, 2014). The use of HBO_2 to treat chronic pain is the subject of ongoing research in both clinical and preclinical research. Clinically, HBO_2 treatment is reportedly effective in a variety of off-label applications, including some chronic pain conditions such as complex regional pain syndrome (formerly reflex sympathetic dystrophy syndrome) (Katznelson et al., 2016; Kiralp et al., 2004; Peach, 1995), fibromyalgia syndrome (Efrati et al., 2015; Yildiz et al., 2004), migraine or cluster headache (Di Sabato et al., 1993; Myers and Myers, 1995; Wilson et al., 1998), myofascial pain syndrome (Kiralp et al., 2009) and idiopathic trigeminal neuralgia (Gu et al., 2012). In the preclinical setting, HBO_2 has been shown to produce long-lasting pain relief in experimental animals (Chung et al., 2010; Gibbons et al., 2013; Warren et al., 1979). However, the mechanism through which HBO_2 produces these effects is not, at present, fully characterized.

Research in our laboratory has focused on elucidating the mechanism of antinociceptive action of HBO_2. We have reported that an 11-min HBO_2 treatment significantly reduced glacial acetic acid-induced abdominal constrictions in mice, an animal model of acute pain (Ohgami et al., 2009; Quock et al., 2011). We also found that repeated daily 60-min HBO_2 treatments for 4 days induced an unparalleled biphasic antinociceptive response that consisted of 1) an early phase that lasted at least 6 h after the HBO_2 treatment before dissipating; and 2) a late phase that emerged about 18 h after the early phase and lasted for up to 3 weeks (Chung et al., 2010). The acute antinociceptive response of mice to HBO_2 is dependent on nitric oxide (NO) (Ohgami et al., 2009). Centrally administered inhibitors of neuronal nitric oxide synthase (nNOS) and antagonists of opioid receptors are able to block HBO_2-induced antinociception in both acute and long-lasting antinociceptive models, indicating a centrally active pathway mediating HBO_2-induced antinociception (Chung et al., 2010; Gibbons et al., 2013; Ohgami et al., 2009; Quock et al., 2011; Zelinski et al., 2009). NO can act as a signaling molecule in the central nervous system and is associated with diverse behaviors including learning, memory and sleeping, as well as sensory and motor function (Garthwaite, 2008). NO has also been shown to function similarly to a neurotransmitter in the CNS depending upon the circuit (Garthwaite, 2008). NO may be produced both pre- and post-synaptically and has been shown to both depolarize and hyperpolarize nerve cells (Garthwaite, 2008). Finally, NO can act as both a neurotransmitter and a neuromodulator by modulating the release of several other neurotransmitters, including GABA (Kuriyama and Ohkuma, 1995).

γ-Aminobutyric acid (GABA) neurons are widely distributed in the central nervous system, including supraspinal and spinal sites involved in transmission of afferent pain signals and descending pain-modulating pathways (Enna and McCarson, 2006). Drugs that activate GABA receptors have been found to produce antinociception in rodents (Krogsgaard-Larsen et al., 2004; Sawynok, 1987). Moreover, GABA receptors have been implicated in mediating stress-induced antinociception (Tokuyama et al., 1992) as well as the antinociceptive effects of drugs such as benzodiazepines (Kunchandy and Kulkarni, 1987), delta opioid peptides and heroin (Rady and Fujimoto, 1996, 1995).

NO has been shown to be necessary for long-term potentiation (LTP) of GABAergic neurons in the amygdala (Lange et al., 2012), the hippocampus (Zhuo et al., 1993), the ventral tegmental area (VTA) (Nugent et al., 2009), and the spinal cord (Fenselau et al., 2011). NO is also known to increase GABA release from presynaptic cells by increasing calcium in the presynaptic cells as well as through the sodium-dependent carrier GABA uptake system (Kuriyama and Ohkuma, 1995). HBO_2-induced antinociception has been shown to be mediated by a NO-sGC-cGMP-PKG pathway (Ding et al., 2017; Quock et al., 2011). The same signaling pathway is involved in NO enhancement of GABA release in the basolateral complex of the amygdala (Lange et al., 2012).

The objective of the present investigation was to assess the possible involvement of GABAergic mechanisms in the acute antinociceptive effect of HBO_2 in a mouse model of acute pain and to provide neuroanatomical evidence for interactions of brainstem nociceptive GABAergic pathways with HBO_2. It is likely that HBO_2-induced acute antinociception is partially mediated by GABA transmission in the spinal cord. HBO_2 can potentially increase NO, which, in turn, increases GABA activity, which contributes to the acute antinociceptive effect of HBO_2.

2. Results

2.1. Drug interaction results

Intrathecal pretreatment with the non-competitive $GABA_A$ antagonist SR 95531 dose-dependently decreased the percent antinociception observed while under HBO_2. A one-way between-subjects ANOVA revealed a main effect of SR 95531 on HBO_2-induced antinociception [$F(4,47) = 10.395$, $P < 0.0001$]. A Dunnett's *post hoc* test was conducted to determine which doses of SR 95531 significantly affected HBO_2-induced antinociception. Animals pretreated with saline vehicle exhibited 56.70 ± 4.4% antinociceptive response to HBO_2. Mice pretreated with 2.5 and 5.0 ng SR 95531 exhibited an antinociceptive effect that held steady at 55.8± 8.6% and 56.1± 6.0%, respectively. The 10-ng dose of SR 95531 decreased HBO_2-induced antinociception to 28.9 ± 4.6% ($P < 0.01$). Furthermore, a 50-ng pretreatment dose further reduced the antinociception to 13.2 ± 6.9% ($P < 0.0001$). Fig. 2 depicts the dose-related antagonism of HBO_2-induced antinociception by 10 and 50 ng SR 95531 when administered directly into the spinal cord.

A one-way ANOVA was conducted to compare the effects of SR 95531 at room air with a vehicle pretreatment at room air. This analysis was not statistically significant [$F(2,30) = 0.7261$, $P > 0.05$], i.e., they were not significantly different from saline vehicle pretreatment

at room air. This indicates that while the 10- and 50-ng doses of SR 95531 are able to antagonize the antinociceptive effect of HBO_2, they have no appreciable effect on their own under room air.

Intrathecal pretreatment with the selective $GABA_B$ receptor antagonist CGP 35348 failed to affect the antinociceptive effect of HBO_2 at all doses tested [$F(3,38) = 0.854$, $P > 0.05$]. Because no effect was found, no multiple comparisons tests were run on CGP 35348. Pretreatment with the highest dose of CGP35348 had no effect on the abdominal constriction test under room air conditions [$t(22) = 0.27$, $p>0.05$]. These results indicate that the $GABA_B$ receptor is not involved in the acute antinociceptive effect of HBO_2 induced by an 11-min treatment of HBO_2. Results of this experiment are shown in Fig. 3.

Intrathecal pretreatment with the GABA uptake inhibitor nipecotic acid increased HBO_2-induced antinociception only at the highest dose used (30 μg). A one-way ANOVA identified an effect of nipecotic acid on HBO_2-induced antinociception [$F(3,49) = 6.974$, $P < 0.005$] (Fig. 4). Multiple comparisons analysis revealed a significant difference between the effects of the vehicle vs. 30 μg of nipecotic acid on HBO_2- induced antinociception ($P < 0.05$). The 30-μg dose of nipecotic acid increased the antinociceptive effect to 75.5 ± 5.2%, compared to the response of the saline vehicle-pretreated group, 44.6 ± 8.7 (Fig. 4). Microinjections of 10 μg and 30 μg nipecotic acid microinjections failed to cause significant change from saline vehicle [$F(2,46)=0.968$ $p>0.05$]. These results indicate that nipecotic acid, a GABA reuptake inhibitor, can increase the antinociceptive effect of HBO_2 if the dose is sufficiently high enough.

2.2. Western immunoblotting results

Densitometric analysis of immunoblots of the $GABA_A$ receptor phosphorylated at the β_3 subunits showed no significant changes in expression of the phosphorylated β_3 subunit. A two-way between-subjects ANOVA found a significant interaction between HBO_2 and glacial acetic acid [$F(1,20) = 8.782$, $P < 0.05$]. However, no main effects of either HBO_2 [$F(1,20) = 0.027$, $P > 0.05$] or glacial acetic acid [$F(1,20) = 0.00017$ $p>0.05$] were found. Expression of the phosphorylated β_3 subunit of the $GABA_A$ receptor decrease in response to either HBO_2 or glacial acetic acid. However, when an animal is administered glacial acetic acid and exposed to HBO_2, expression significantly increases in the lumbar region of the spinal cord. This indicates a significant interaction between the presence of the nociceptive stimulus, in this case, glacial acetic acid, and HBO_2 on phosphorylation of the β_3 subunit of the $GABA_A$ receptor in the lumbar spinal cord (Figs. 5A and 5B).

Densitometric analysis indicates a reduction in expression of nNOS 11 min following injection with glacial acetic acid in the lumbar spinal cord when compared to animals injected with saline and sacrificed at the same time (Figs. 6A and 6B). A twoway ANOVA was run on the densitometric analysis data and revealed a significant interaction between HBO_2 and glacial acetic acid as measured by nNOS expression [$F(1,20) = 5.748$, $P < 0.05$]. No main effects were found for either glacial acetic acid [$F(1,20) = 2.67$, $P > 0.05$] or HBO_2 [$F(1,20) = 0.297$, $P > 0.05$]. This indicates that nNOS expression decreased in response to HBO_2 when saline, rather than glacial acetic acid, was administered. nNOS expression also decreased in response to glacial acetic acid, but this effect alone was not significant. This

would mean that in the absence of a noxious stimulus, HBO_2 would decrease nNOS expression. However, when HBO_2 was administered to animals after glacial acetic acid, nNOS expression increased in the lumbar region of the spinal cord.

Analysis of the expression of the phosphorylated β_3 subunit of the $GABA_A$ receptor showed an interaction between HBO_2 and glacial acetic acid. A two-way between-subjects ANOVA found no significant interaction [$F(1,20) = 1.060$, $P > 0.05$]. There was no main effect of HBO_2[$F(1,20) = 3.161$, $P > 0.05$], and there was also no main effect of glacial acetic acid [$F(1,20) = 0.47$, $P > 0.05$]. This indicates that neither HBO_2 nor glacial acetic acid significantly affected expression of the β_3 subunit of the $GABA_A$ receptor in the thoracic region of the spinal cord and that there was no significant interaction between the two at this level of the spinal cord.

A two-way between-subjects ANOVA revealed no interaction between HBO_2 and glacial acetic acid in expression of nNOS in the thoracic spinal cord [$F(1,20) = 0.146$, $P > 0.05$]. There was also no main effect of either HBO_2 [$F(1,20) = 0.05$, $P > 0.05$], or glacial acetic acid [$F(1,20) = 0.77$, $P > 0.05$] in density of nNOS as compared to density of GAPDH across the different conditions of animal exposure. This indicates that neither HBO_2 nor glacial acetic acid affected nNOS expression in the thoracic region of the spinal cord.

3. Discussion

The purpose of this study was to investigate a possible role for spinal cord GABA in a model of HBO_2-induced acute antinociception using a combination of pharmacological, behavioral and molecular biology methods. The $GABA_A$ antagonist SR 95531 attenuated HBO_2-induced acute antinociception in a dose-dependent manner at very low doses (nanomolar range), thus implicating $GABA_A$ receptors in HBO_2-induced antinociception. We were also able to enhance HBO_2-induced antinociception by pretreating with nipecotic acid, an inhibitor of the GABA reuptake transporter (Johnston et al., 1979). This indicates that the synaptic levels of GABA are positively correlated with the magnitude of the HBO_2-induced acute antinociceptive effect.

A role for the $GABA_B$ receptor was discounted as the doses of CGP 35348 used failed to affect the antinociceptive effect of HBO_2. CGP 35348 alone also produced no change in abdominal constrictions. We conclude that spinal $GABA_B$ receptors are not involved in HBO_2-induced acute antinociception in mice, although it is possible that the doses used were too low to influence the antinociceptive effect of HBO_2. However, CGP 35348 doses comparable to ours administered into the mouse lateral cerebral ventricle antagonized (±)-baclofen-induced antinociception in the abdominal constriction test (Malcangio et al., 1991). It is possible that this dose is not high enough when administered into the thecal space compared to ventricular space. Unpublished data from our lab indicates that this might not be the case as intracerebroventricular administration of the same doses of CGP35348 also failed to block HBO_2. This evidence supports the supposition that antagonism of the $GABA_B$ receptor does not affect antinociception under HBO_2.

Previously, we demonstrated that the antinociceptive effect of an 11-min HBO_2 treatment was modulated in part by both NO and opioid mechanisms (Heeman et al., 2013; Ohgami et al., 2009; Quock et al., 2011). Our current results indicate that acetic acid decreases nNOS expression in the lumbar spinal cord and this effect is not as prevalent in animals treated with HBO_2. In another study, it was observed that a 60-min treatment of HBO_2 increased levels of NO oxidation products in the spinal cord of adult rats (Ohgami et al., 2008). Furthermore, the involvement of NO was verified by dose-related antagonism of HBO_2-induced antinociception by i.t. administered nitric oxide synthase (NOS) inhibitors and attenuation of the HBO_2 effect by using nNOS knockout mice and antisense oligodeoxynucleotides to nNOS to knock down NOS (Ohgami et al., 2009). Our current results indicate that GABA acting at the $GABA_A$ receptor in the spinal cord may also be involved in the HBO_2 induced antinociception. Our current results do not conclusively link HBO_2 effects on nNOS to effects HBO_2 may have on GABA although previous studies have indicated that nNOS and may influence GABAergic signaling. GABA localization studies indicate the presence of many GABAergic interneurons in the spinal cord, especially in the superficial lamina (Sardella et al., 2011). In particular, co-localized nNOS- and GABA-immunoreactive neurons have been found in lamina I and lamina II, and nNOS was found in terminal boutons containing vesicular GABA transporters (Sardella et al., 2011). Therefore, this is a possible pathway in which GABA may interact with the NO-sGC-cGMP pathway, which has been implicated in HBO_2-induced antinociception (Ding et al., 2017; Quock et al., 2011).

Isoflurane administration during i.t. procedures can complicate the interpretation of our results since isoflurane has been shown to have both neuroprotective and neurotoxic affects (Jiang et al., 2017). Isoflurane pretreatment has been implicated as having protective effects in ischemia in animal models (Jiang et al., 2017). In animal models of neural injury in neonatal rats, isoflurane was shown to decrease the hypoxia-related increase in the release of amino acid neurotransmitters while elevating levels of GABA (Zhao et al., 2016). However, in the above experiment, animals were exposed to varying concentrations of isoflurane for a prolonged period — 6 hours (Zhao et al., 2016). In our experiments, animals were exposed to isoflurane at a concentration of 2.5% isoflurane for no more than 5 min at a time. In addition, all our animals receiving i.t. injections received isoflurane so any effect of isoflurane on GABA concentrations would be consistent across all animals receiving i.t. injections.

It should be noted, however, there are other possible explanations for these results. A previous study using a chronic constriction injury (CCI) model of neuropathic pain found that HBO_2 was effective at decreasing CCI induced allodynia (Fu et al., 2017). Furthermore, while CCI increased apoptosis of GABAergic neurons in the dorsal horn, HBO_2 administration inhibited this effect which protects the balance between excitation and inhibition in the spinal cord (Fu et al., 2017). The results of this paper implicate a neuroprotective effect of HBO_2 on GABAergic neurons in the dorsal horn of the spinal cord in relief of neuropathic pain (Fu et al., 2017). While our experiments did not investigate neuroprotective effects of HBO_2, they do implicate GABA as having a role in HBO_2-induced antinociception which is in agreement with the prior experiment. HBO_2-induced antinociception could be caused by interactions between the opioidergic and GABAergic

pathways that are induced by changes cause by HBO_2. Still other possibilities could include supraspinal serotonergic and/or noradrenergic pathways that recruit local GABAergic inhibitory neurons in the spinal cord (Millan, 2002). These data indicate that the pathways involved in HBO_2-induced antinociception are complex and interactive. More research is needed to distinguish exactly how various pathways interact in HBO_2 induced antinociception.

The western blotting results indicate an interaction between the noxious stimulant for abdominal constrictions, glacial acetic acid, and HBO_2 in expression of the phosphorylated β_3 subunit of the $GABA_A$ receptor in the lumbar but not thoracic spinal cord. Glacial acetic acid and HBO_2 independently decreased expression of the pβ_3 subunit of the $GABA_A$ receptor; however, in the presence of both, expression of the $GABA_A$ receptor subunit was restored to control levels. Isoflurane was not given to animals involved in western blotting eliminating the possibility that elevated GABA levels related to isoflurane exposure could explain these results (Zhao et al., 2016). This could indicate that, in the presence of an acute pain stimulus, expression is reduced, but this decrease is reversed by HBO_2. In contrast to the $GABA_A$ receptor, HBO_2 did not fully restore nNOS expression in the spinal cord to control levels. In the presence of noxious stimulation, nNOS expression in the spinal cord is significantly decreased. HBO_2 did slightly increase expression of nNOS in the lumbar spinal cord in the presence of pain, but this effect was not statistically significant. HBO_2 alone itself decreased nNOS expression compared to control, and this is not significantly affected by the presence or absence of noxious stimulation, although the reduction in nNOS in the lumbar spinal cord was not as great as that in the presence of pain. These results indicate that, in the lumbar spinal cord, HBO_2 increases the expression of the phosphorylated β3 subunit of the $GABA_A$ receptor. We focused on this receptor because phosphorylation at this site could indicate increased activity of the receptor (McDonald et al., 1998). Thus, restoration of phosphorylation of this site by HBO_2 and painful stimuli could indicate that GABA activity at the $GABA_A$ receptor in lumbar spinal cord is restored when both HBO_2 is producing antinociception. However, this effect is not dependent upon nNOS pathways because, while nNOS expression is also decreased when pain is present, it is not restored by HBO_2.

We hypothesize that HBO_2 increases GABA activity at the $GABA_A$ receptor via a nitric oxide dependent mechanism. Blotting for the phosphorylated β_3 subunit of the $GABA_A$ receptor should theoretically be increased when there is more GABA activity at the receptor (McDonald et al., 1998). We expected that more phosphorylation at the $GABA_A$ receptor would occur after a pain stimulus due to increased activation of inhibitory post-synaptic processes at the lumbar level of the spinal cord. An increase in phosphorylation at the β_3 subunit has been linked to both increased and decreased receptor function of the $GABA_A$ receptor (Brandon et al., 2000; McDonald et al., 1998). This effect may be partially caused by the protein kinase that phosphorylated the subunit. Specifically, a major site of regulation on the β_3 subunit of the $GABA_A$ receptor is the serine 408/409 phosphorylation site. PKA can differentially regulate phosphorylation to either increase activation or decrease activation based on whether both serines 408 and 409 are phosphorylated (potentiation of $GABA_A$ activity) or whether just 409 is phosphorylated (inhibition of $GABA_A$ receptor activity) (McDonald et al., 1998). Our antibody was specific for phosphorylation of both serine 408

and 409 so it is reasonable to infer that a decrease in expression can be linked to inhibition of activity and an increase (or restoration) can be linked to potentiation of $GABA_A$ receptor activity. Our results support the conclusion that glacial acetic acid and HBO_2 separately inhibit $GABA_A$ receptor activity. However, when combined, HBO_2 reversed the decreased activity. These results support our behavioral finding that blockade of the $GABA_A$ receptor reduces antinociceptive activity of HBO_2 in the acetic acid test.

Glacial acetic acid caused a decrease in nNOS expression that was partially prevented by HBO_2. This supports earlier findings from our lab that blocking NO could antagonize the antinociceptive effect of HBO_2 because NO release being at levels seen prior to the pain stimulus would be required for antinociception under HBO_2 (Ohgami et al., 2009; Quock et al., 2011). We expected HBO_2 to further increase this level and that this would coincide with an increase in nNOS levels. Similarly, we expected nNOS expression to be increased after HBO_2. While nNOS expression was higher under HBO_2 when the animals were exposed to glacial acetic acid, levels of expression were decreased, although not significantly, compared to control. In neuropathic and inflammatory pain models, NO is hypothesized to play a role in central sensitization (Wu et al., 2001). However, NO has also been found to have an antinociceptive role in pain (Schmidtko et al., 2009).

Our results could indicate that, while HBO_2 does prevent or decrease expression of the phosphorylated β_3 subunit of the $GABA_A$ receptor in the presence of a noxious stimulus, this effect does not coincide with an increase in nNOS above normal levels. Therefore, we cannot conclude that the effects of antinociceptive effects of HBO_2 involve a pathway involving both nNOS and GABA. However, it should be important to note that we only blotted for one subunit of the $GABA_A$ receptor and our results might not hold for phosphorylation at any of the other receptor subunits. In addition, we may have a low density of β_3 subunits in the lumbar spinal cord. This is not likely because immunohistochemical studies have found that β_3 subunits are widespread in the dorsal horn of the spinal cord, although not in the motor neurons (Bohlhalter et al., 1996). We also did not examine whether either of the other two NOS isoforms—endothelial NOS (eNOS) or inducible NOS (iNOS)—might increase under these same conditions. If so, this might compensate for the lower amounts of available NO from nNOS. Indeed, prior studies with knockout mice indicate that this is likely as inhibition of any one of the three isoforms can result in a compensatory increase in the other two isoforms (Boettger et al., 2007). It is also possible that we did not wait sufficiently long for major changes in nNOS expression to occur. Since nNOS is post-transcriptionally regulated, it may take longer than the time elapsed in this study for significant changes to occur so we may not be collecting samples at the time of maximum change. Indeed, changes in nNOS levels and NO activity were generally seen at later time points in previous studies. The maximum time varies, but other studies have found it to be at least 30 min after injections of acetic acid (Larson et al., 2000; Shi et al., 2005; Wu et al., 2001). Animals in this study were sacrificed immediately after decompression and 30 min did not elapse from the time of the initial injection to the time of sacrifice.

In the thoracic spinal cord, neither glacial acetic acid nor HBO_2 had an effect on nNOS expression or expression of the phosphorylated β_3 subunit of the $GABA_A$ receptor. This

result was not unexpected due to the preponderance of nociceptive signaling in the lumbar versus thoracic spinal cord. In addition, the acetic acid test is not necessarily a valid model of visceral pain due to specificity problems with the model; for example, non-analgesics have been found to have antinociceptive activity according to the model, and the abdominal constrictions include the muscles of the abdominal wall rather than just the organs of the viscera (Langford and Mogil, 2011; Ness, 1999). Despite this criticism, many laboratories use the acetic acid test as a model of visceral pain because it has been a very powerful predictive tool and has a unique ability to detect weaker antinociceptive drugs (Langford and Mogil, 2011). Indeed, some labs use the acetic acid test as an assay of chemical nociception rather than a model of visceral pain (Lariviere et al., 2002; Spindola et al., 2012). Our study found that an injection of glacial acetic acid into the peritoneal cavity had no effect on protein expression in the spinal cord. In other pain models such as the formalin and capsaicin models, nNOS expression has been shown to increase in response to noxious insult in the spinal cord (Shi et al., 2005; Wu et al., 2001).

4. Conclusions

We found evidence that the $GABA_A$ receptor is involved in HBO_2-induced antinociception because HBO_2-induced acute antinociception was antagonized in a dose-dependent manner by the selective competitive $GABA_A$ antagonist SR 95531. Preventing GABA reuptake using the GAT inhibitor nipecotic acid increased the antinociceptive response to an 11-min treatment of HBO_2. The $GABA_B$ antagonist CGP 35348 failed to antagonize HBO_2-induced antinociception at the doses used. HBO_2 did restore expression of the phosphorylated β3 subunit of the $GABA_A$ receptor in the presence of acetic acid. It is possible that the antinociceptive effects of HBO_2 are caused by preventing or restoring activity of GABAergic inhibitory interneurons in the lumbar region of the spinal cord. We were unable to conclusively link this effect to NO system because HBO_2 failed to restore nNOS expression in the presence of acetic acid or by itself.

5. Experimental Procedure

5.1. Animals

A total of 221 Male NIH Swiss mice, 24–26 g, were used in these experiments (Harlan Laboratories, Indianapolis, IN). Animals were group-housed (4 per cage) in standard mouse cages (11.5 cm × 16.5 cm × 28.0 cm) and kept under standard laboratory conditions (22 ± 1°C room temperature, 33% humidity) on a 12-h light cycle from 0600 to 01800 h. All animals were housed in the Animal Resource Unit and Wegner Hall Vivarium of Washington State University, which are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals received food and water *ad libitum*. Mice were kept in the holding room for four days after arrival in the facility for acclimation prior to experimentation. All procedures were approved by the Washington State University Institutional Animals Care and Use Committee (IACUC) and subject to post-approval review and carried out in accordance with *The Guide for the Care and Use of Laboratory Animals, 8th Edition* (National Academies Press, Washington, DC, 2011). All measures to minimize pain or discomfort were taken by the investigators.

5.2. HBO_2 treatment

A single cage of four mice was placed in a B-11 research hyperbaric chamber (Reimers Systems Inc., Lorton, VA) following i.p. injection with glacial acetic acid. The B-11 research chamber consists of a cylindrical clear acrylic chamber (27.9 cm diameter × 55.9 cm L). The chamber was ventilated with 100% oxygen (O_2), U.S.P. (AL Compressed Gases Inc., Spokane, WA) at a flow rate of 20 L/min. The pressure inside the chamber was increased to the desired pressure of 3.5 atmospheres absolute (ATA) over a period of about two min. The mice breathed normally during the HBO_2 treatment. The pressure was held at 3.5 ATA for about three min prior to the start of the 6-min observation period. After completion of the HBO_2 treatment, animals were decompressed over the next 2–3 min.

5.3. Abdominal constriction test

Antinociception was assessed using the abdominal constriction test (Siegmund et al., 1995). Briefly, mice were injected i.p. with 0.1 ml/10 g body weight of 0.6% glacial acetic acid. After 5 min, the number of abdominal constrictions—lengthwise stretches of the torso with concave arching of the back—were counted for the next 6 min. The experimenter was not blinded to treatment condition; however, some vehicle controls were run simultaneously for direct comparison of drug group and vehicle controls. Calculation of the degree of antinociception was computed using the formula below:

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

5.4. Intrathecal (i.t.) microinjections

I.t. pretreatments were made using the microinjection technique of Hylden and Wilcox (1980). Briefly, mice were anesthetized with 2% isoflurane in oxygen in an anesthesia chamber or with a nosecone during microinjection. The animal was held by the pelvis with the hind legs extended backward. The microinjection was made through the skin into the spinal cord. A luer-tipped 10-μl microsyringe was fitted with a ½-inch, 30-gauge disposable needle. This needle was then inserted between the lumbar vertebrae below L_6. The volume of solution injected i.t. was 5.0 μl delivered over 30 s. Experiments only proceed when the experimenter has achieved >75% accuracy. In addition, experimenters use the hips as an external landmark and hold the hips of the mouse firmly while completing the microinjection. Once the intrathecal space has been penetrated, foot and tail reactions are sought before proceeding with the injection. Intrathecal microinjections occurred 25 min prior to acetic acid injections so that assessment began 30 min following pretreatment. Animals were placed into the hyperbaric chamber immediately following the glacial acetic acid injections. A timeline of these experiments can be seen in figure 1A.

5.5. Drugs

Three drugs were used in this study: 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide (SR 95531 – a $GABA_A$ receptor antagonist); (3-aminopropyl)-(diethoxymethyl)-phosphinic acid (CGP 35348 – a $GABA_B$ receptor antagonist); and (±)-3-piperidine carboxylic acid [(±)-nipecotic acid – a GABA transporter

inhibitor]. All three drugs were purchased from Tocris Biosciences (a subsidiary of Bio-Techne, Minneapolis, MN). All drugs were freshly prepared in 0.9% physiological saline prior to administration by i.t. microinjection. The doses were derived from the published literature (Georgiev et al., 1995; Malcangio et al., 1991; Song et al., 1998). Fifty-one mice were randomly assigned to be treated with either saline vehicle or one of four doses of SR95531. Thirty min after the i.t. injection, the animals were injected with acetic acid and immediately exposed to HBO_2. An additional 33 mice were given an i.t. injection of either saline vehicle or one of the two highest doses of SR95531. Twenty-nine mice were randomly assigned to be injected with one of three doses of CGP 3534. All of these mice were injected with acetic acid 30 min after i.t. injection and immediately treated with HBO_2. An additional 8 mice were given an i.t. injection of either saline vehicle or the highest dose of CGP 35348 30 min prior to room air; these mice were treated with room air and were used as a control. Finally, 53 mice received either saline or a randomly selected dose of nipecotic acid and exposed to HBO_2 while undergoing the acetic acid test. An additional 20 mice were given an i.t. injection of the two highest doses of nipecotic acid or vehicle.

5.6. Lysate preparation

A separate group of 24 mice was randomly assigned to receive either saline or acetic acid injections. Immediately following injections, mice were treated with either room air or HBO_2. Immediately following cessation of HBO_2 treatment (or exposure to room air), animals were sacrificed and spinal cords were removed by forcible injection of saline into the spinal column and recovered from the decapitated end of the column. A time line of these experiments can be seen in figure 1B. Spinal cords were dissected on ice and all but the lumbar and thoracic regions of the spinal cord were discarded. A volume of 1000 μl of ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer, containing commercially available phosphatase and protease inhibitor cocktails (Biotool.com, Houston, TX), was added to a sample of about 5.0 mg of spinal cord. This volume was chosen because of the high concentration of protein. Tissues were homogenized with a manual homogenizer. The homogenate was centrifuged for 20 min at 4°C. The supernatant was transferred to a fresh tube kept on ice, and the pellet was discarded.

5.7. Preparation of samples

Protein concentration of the homogenized spinal cord was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific Pierce, Rockford, IL) and a Synergy Neo Plate reader with Genmate software (BioTek Instruments Inc., Winooski, VT). All protein concentrations were determined by optical density and compared to a standard curve of BSA (bovine serum albumin) in accordance with the BCA kit instructions.

After protein quantification, samples were prepared for western blotting by adding loading buffer to samples. Sixty μg protein were added to each well as follows: Pre-stained protein ladder, control, acetic acid, acetic acid + HBO_2, and HBO_2 on each gel.

5.8. Western immunoblotting

Protein samples were run on a 10% poly-acrylamide SDS PAGE gel. The gel was inserted into the tank and then run at 95 volts for 120 min. The protein was then transferred to

nitrocellulose paper at a current of 350 mA for 60 min. The membranes were blocked in 5% bovine serum albumin (BSA) in a mixture of Tris-buffered saline and Tween 20 (TBS-T) for 60 min at room temperature. After blocking, the nitrocellulose paper was cut at about 120 kDA, 90 kDA and 40 kDA, using the pre-stained protein ladder so that the standard protein concentrations could be compared to the protein of interest. The separate pieces of nitrocellulose were washed briefly with TBS-T.

Following the washing steps, the primary antibodies for phosphorylated $GABA_A$ Rβ3 antibody (P1130–4089, PhosphoSolutions, Aurora, CO; NBP-2508, Novus Biologicals, Littleton, CO), nNOS (4231S, Cell Signaling, Danvers, MA), and glyceraldehyde-3-phospahte dehydrogenase (GAPDH) (PA1–988, ThermoFisher Scientific, Grand Island, NY; A300–641A-T, Bethyl Laboratories Inc., Montgomery, TX) were added to the membrane piece containing their corresponding proteins. All primary antibodies were generated in rabbit and diluted to a concentration of 1:1000 in 1% BSA in TBS-T. Primary antibody was allowed to sit on the membrane overnight at 4°C with gentle agitation. The following morning, membrane pieces were washed three times for 10 min in TBS-T. Secondary anti-rabbit antibody (7074S, Cell Signaling, Danvers, MA) conjugated to horseradish peroxidase was applied to each membrane piece for 120 min at room temperature at a concentration of 1:1000 in TBS-T. The membrane pieces were washed again three times at 15 min per wash in TBS-T. Supersignal chemiluminescent substrate (Thermo Scientific) was added to each membrane piece, and the pieces were exposed to fluorescent light using a Chemidoc Imaging System (Biorad, Hercules, CA). Finally, relative expression was analyzed by densitometry using Chemidoc software.

5.9. Statistical analysis of data

Percent changes in antinociception were analyzed using a univariate analysis of variance (ANOVA) test for each drug. Where significant differences were found, a *post hoc* Dunnett's multiple comparison test was used to assess HBO_2-induced antinociception between groups. For room air controls, univariate ANOVA tests were run for two of the drugs, but one of the antagonists only had two groups at room air so an unpaired t-test was run for this group to make a pairwise comparison. The α-level was set at 0.05 to protect against type I error.

A multivariate ANOVA was used to compare GABA/GAPDH and nNOS GAPDH expression between groups. A *post hoc* Tukey's test was used for multiple comparisons between groups.

Acknowledgements

This research was supported by NIH Grant AT-007222, the Allen I. White Distinguished Professorship from Washington State University, the Chico Hyperbaric Center (Chico, California), and the Chinese Scholarship Council (Joint Doctoral Program).

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Highlights

- HBO_2-induced antinociception is mediated by spinal $GABA_A$ but not $GABA_B$ receptors.
- Acetic acid reduced expression of nNOS and $GABA_A$ β3pSER 408/409.
- HBO_2 treatment restored nNOS and $GABA_A$ β3pSER 408/409 expression to control levels.
- Protein expression was altered in lumbar but not thoracic spinal cord.

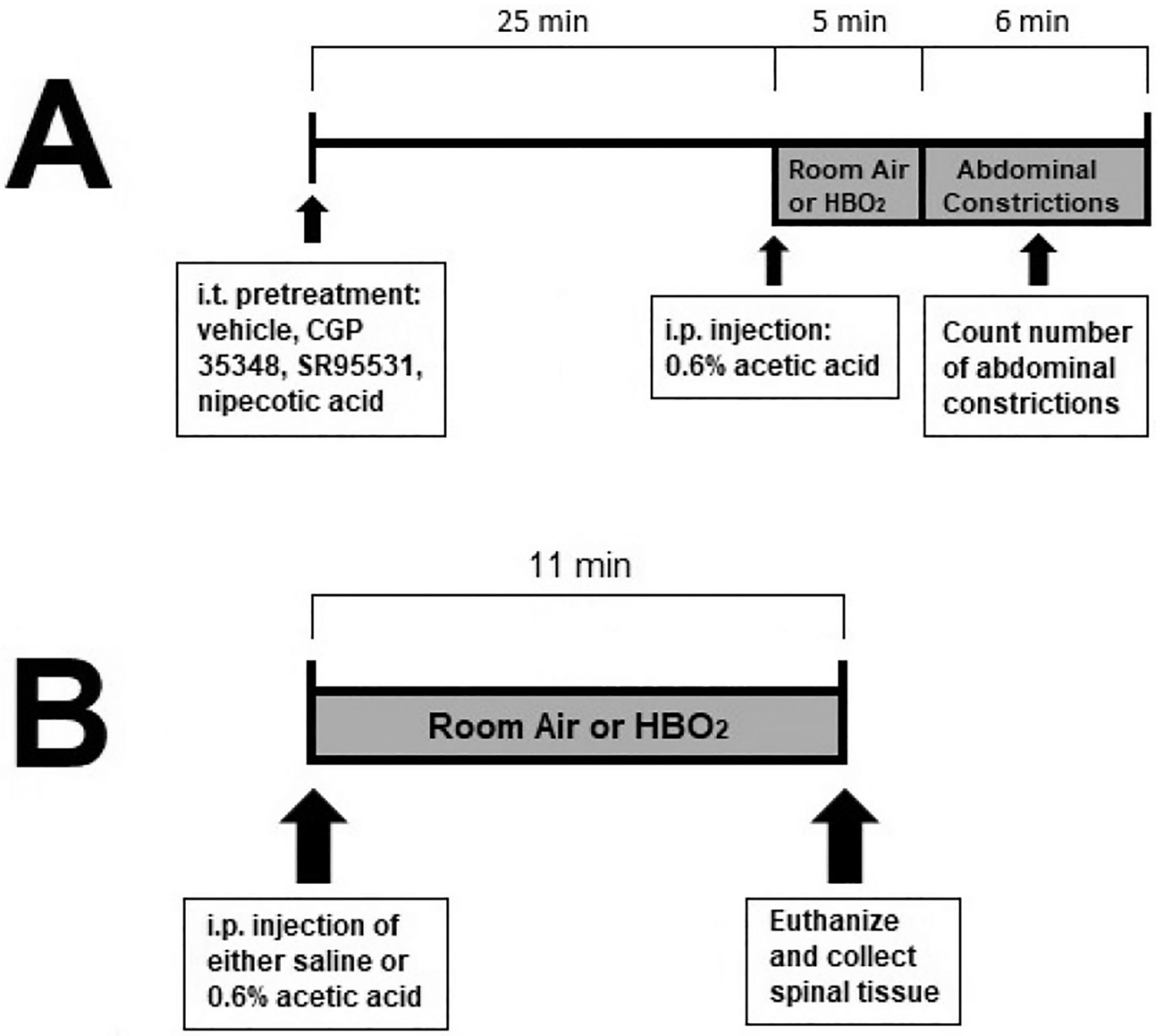

Fig. 1.

Timeline of experiments. **(A)** depicts the typical time course of i.t. pretreatment experiments to investigate the effect of antagonist pretreatment on the antinociceptive effect of HBO_2. Animals were treated with one of four possible treatments: Saline vehicle, SR 95531, CGP 35348, and nipecotic acid, on the antinociceptive effect of HBO_2. **(B)** Depicts the time course of tissue collection for western blot experiments.

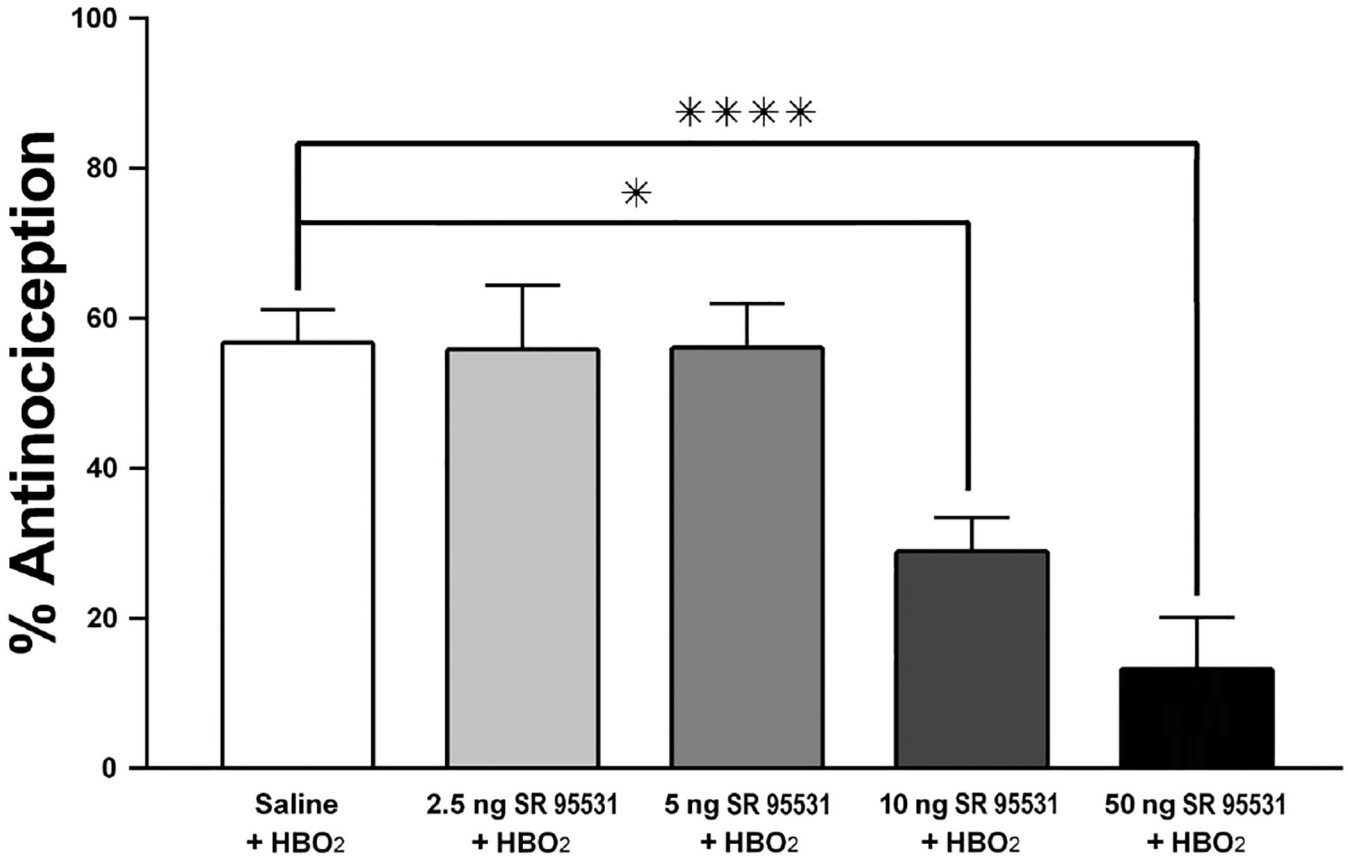

Fig. 2.

Influence of i.t. pretreatment with a $GABA_A$ receptor antagonist (SR 95531)on the acute (11-min) antinociceptive effect of HBO_2 in the glacial acetic acid-induced abdominal constriction test. Each column represents the percent antinociceptive response ± S.E.M. of at least 10 mice per group. Significance of difference: *, $P < 0.05$ and ****, $P < 0.0001$, compared to the saline-pretreated control group.

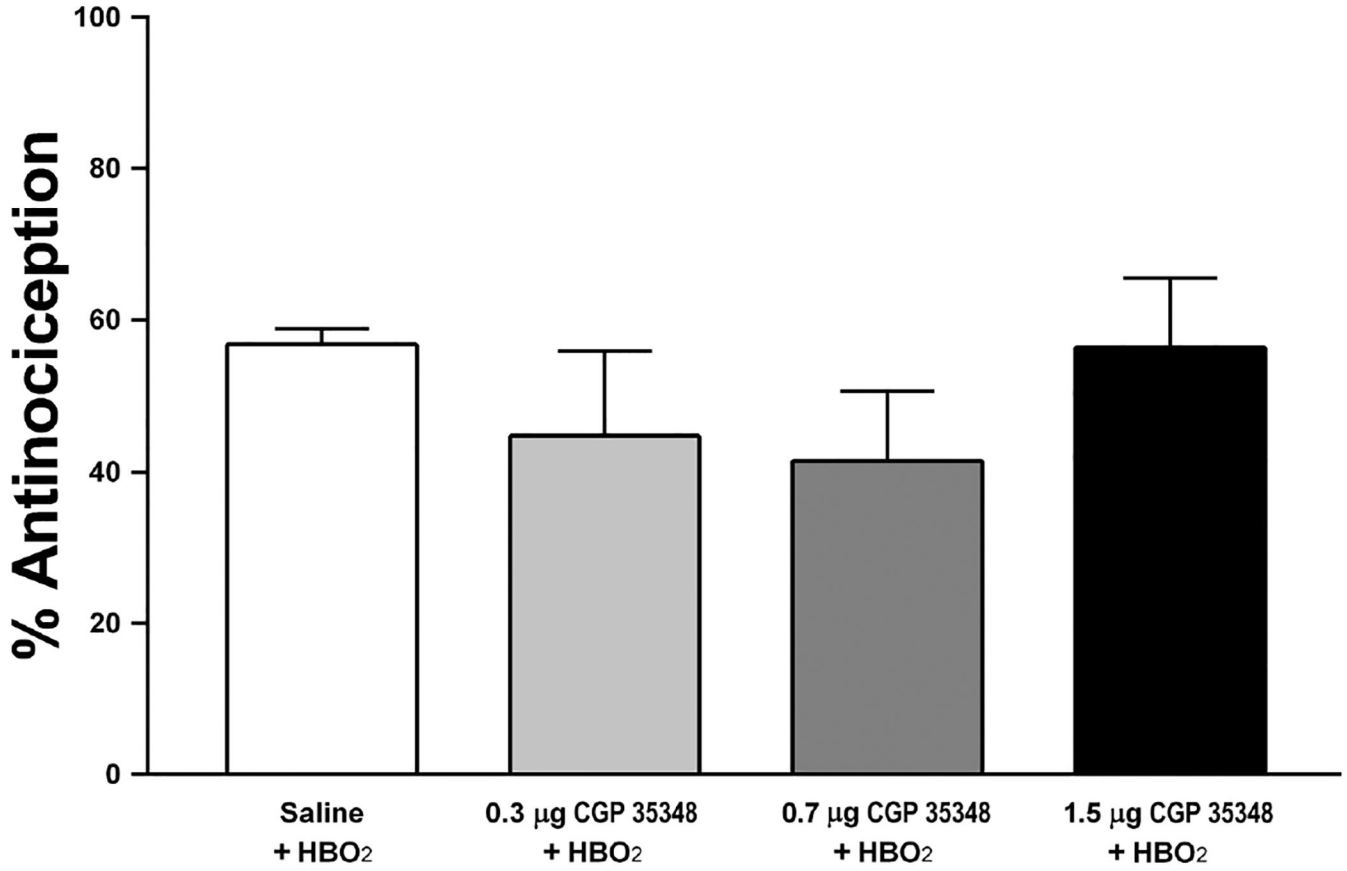

Fig. 3.

Influence of i.t. pretreatment with a $GABA_B$ receptor antagonist (CGP 35348) on the acute (11-min) antinociceptive effect of HBO_2 in the glacial acetic acid-induced abdominal constriction test. Each column represents the percent antinociceptive response ± S.E.M. of at least 10 mice per group. There were no statistically significant differences among the groups.

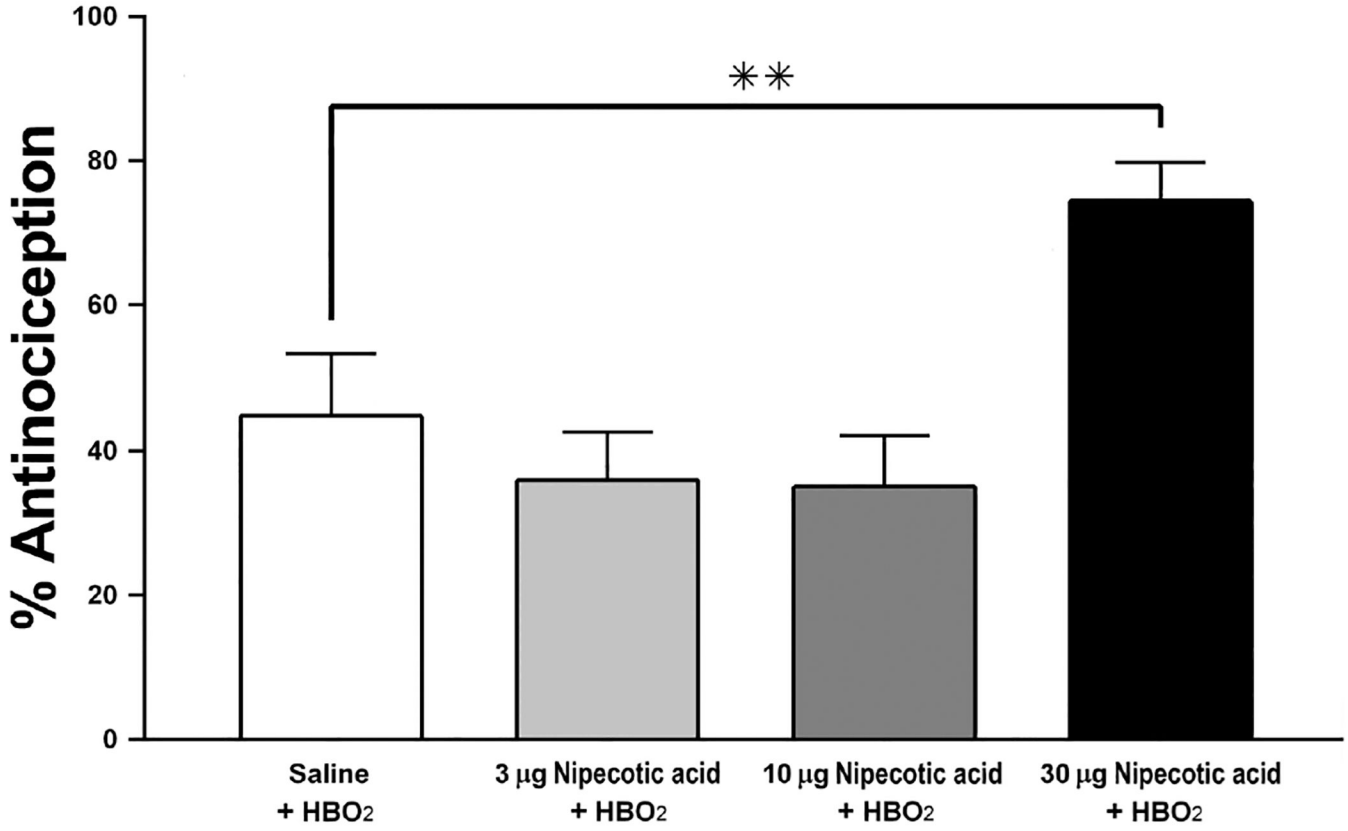

Fig. 4.

Influence of i.t. pretreatment with a GABA uptake inhibitor (nipecotic acid) on the acute (11-min) antinociceptive effect of HBO_2 in the glacial acetic acid-induced abdominal constriction test. Each column represents the percent antinociceptive response ± S.E.M. of at least 10 mice per group. Significance of difference: **, $P < 0.01$, compared to the saline-pretreated control group.

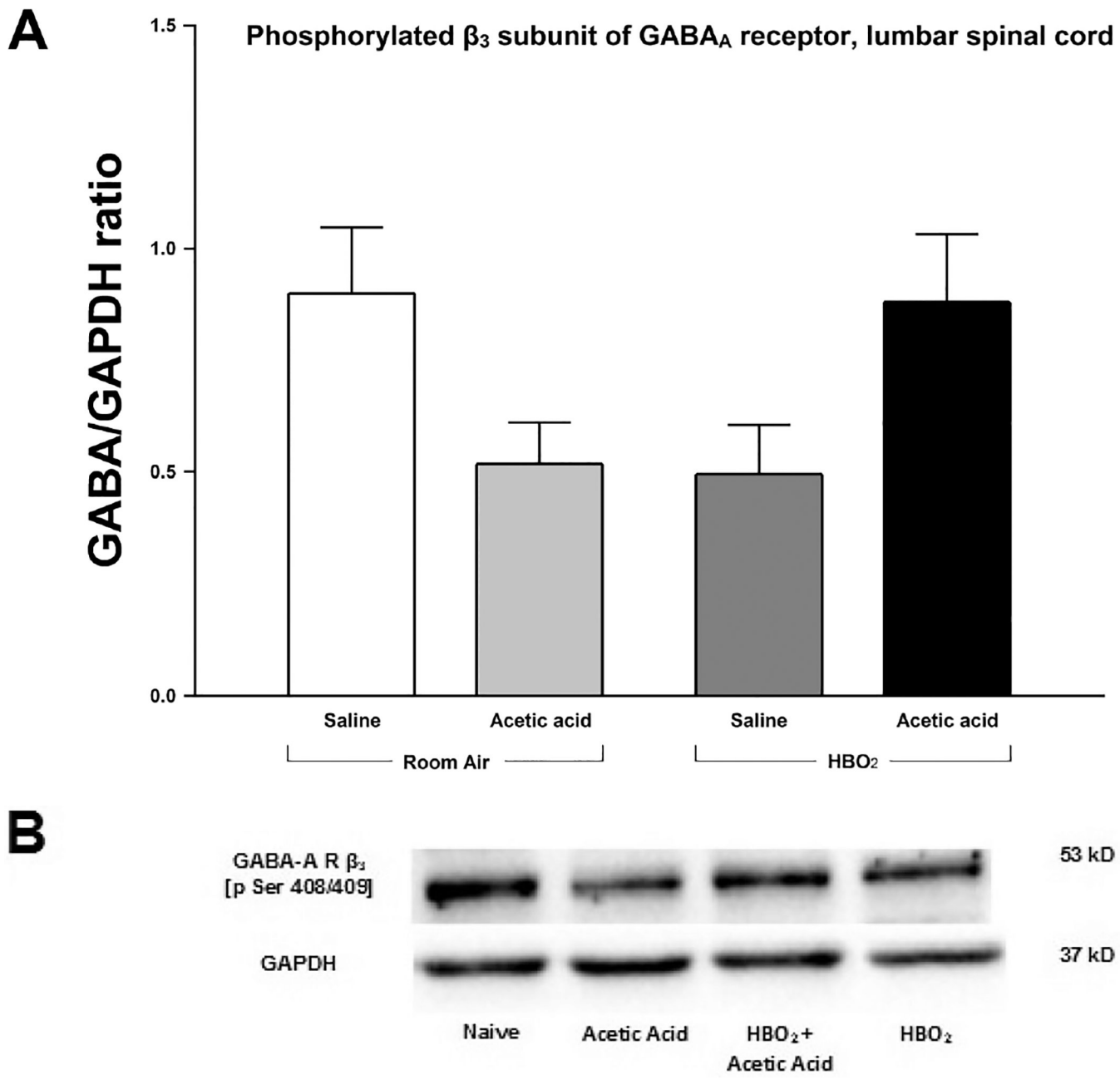

Fig. 5.

(**A**) Effect of glacial acetic acid and HBO_2 treatment on the optical density of phosphorylated β_3 subunit of the $GABA_A$ receptor in the lumbar section of the spinal cord. Each column represents the ratio compared to naïve of $GABA_A$ β3 Pser408/409 to GAPDH ratios of 6 western blots representing 2 separate samples per group. No significant differences were found. (**B**) Representative example of typical western blot of $GABA_A$ β3 Pser408/409 and GAPDH controls.

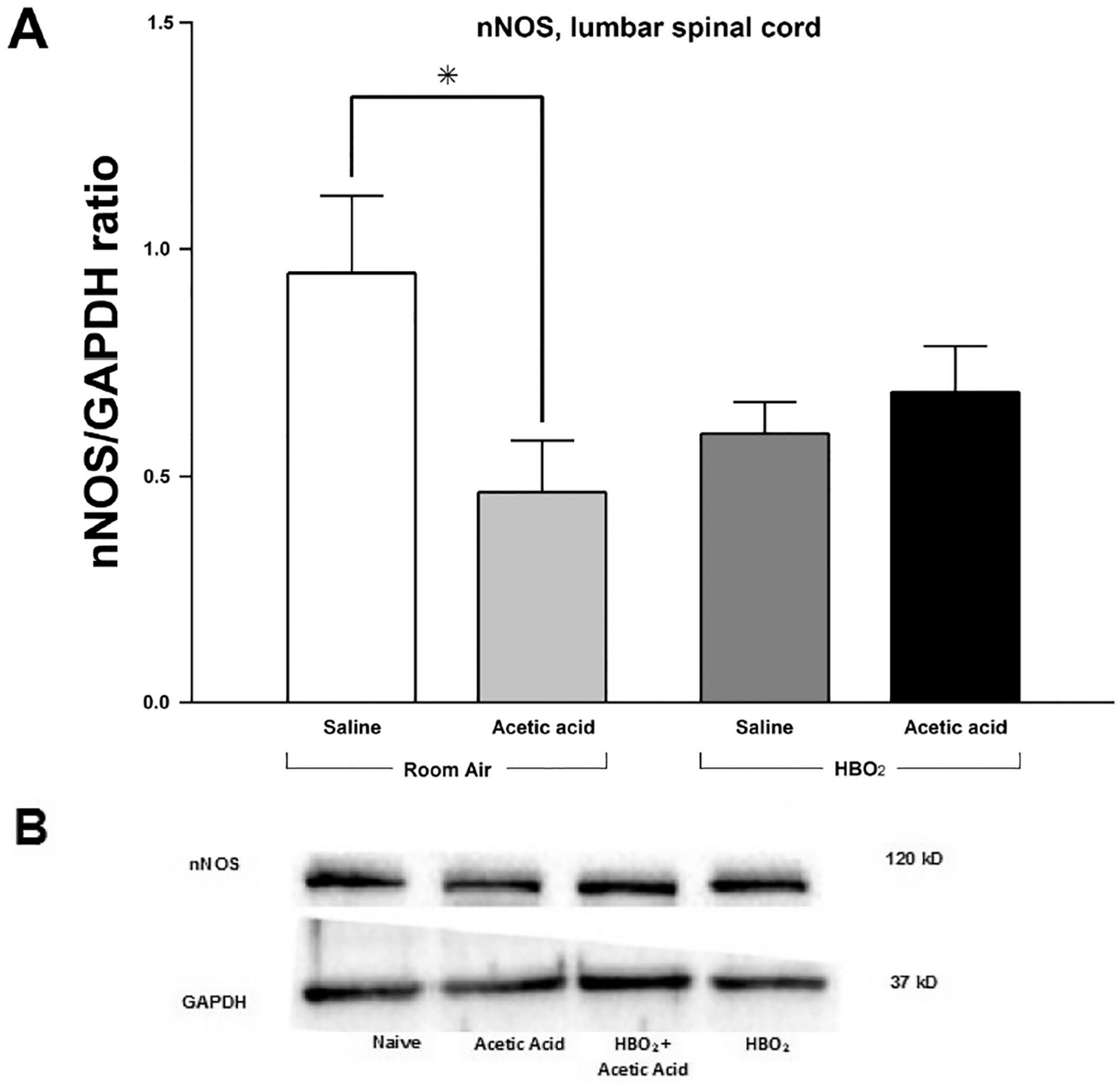

Fig. 6.

(**A**) Effect of glacial acetic acid and HBO_2 treatment on optical density of neuronal nitric oxide synthase (nNOS) in the lumbar section of the spinal cord. Glacial acetic acid causes a decrease in optical density that HBO_2 is able to reverse. Each column represents the ratio compared to naïve of nNOS to GAPDH ratios of 6 western blots representing at least 2 separate samples. Significance of difference: *, $P<0.05$. (**B**) Representative example of western blot of lumbar spinal cord showing expression of nNOS and GAPDH.

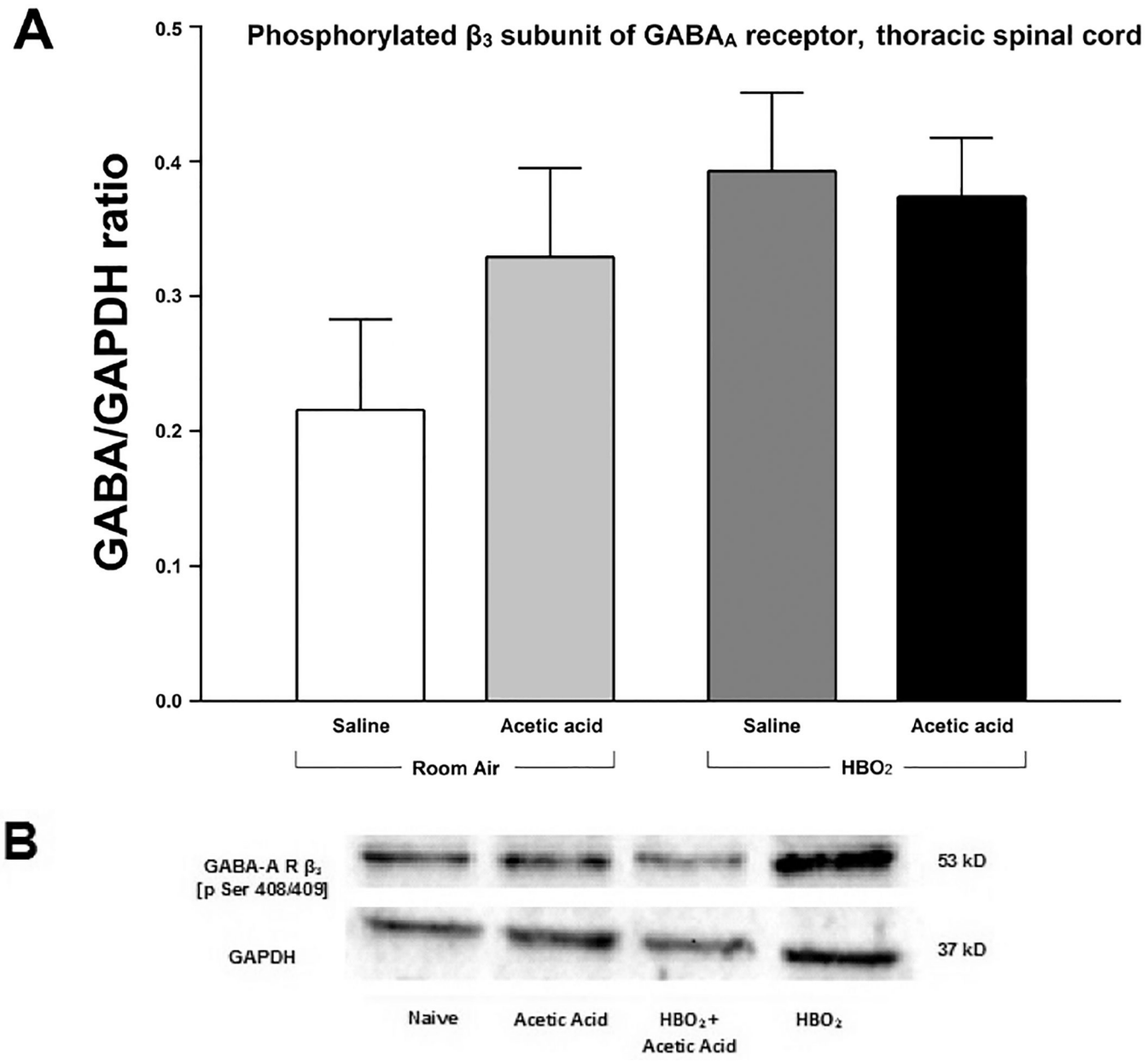

Fig. 7.

(**A**) Effect of glacial acetic acid and HBO_2 treatment on the optical density of phosphorylated β_3 subunit of the $GABA_A$ receptor in the thoracic section of the spinal cord. Each column represents the ratio compared to naïve of $GABA_A$ β3 Pser408/409 to GAPDH ratios of 6 western blots representing 2 separate samples per group. (**B**) Representative example of typical western blot of $GABA_A$ β3 Pser408/409 and GAPDH.

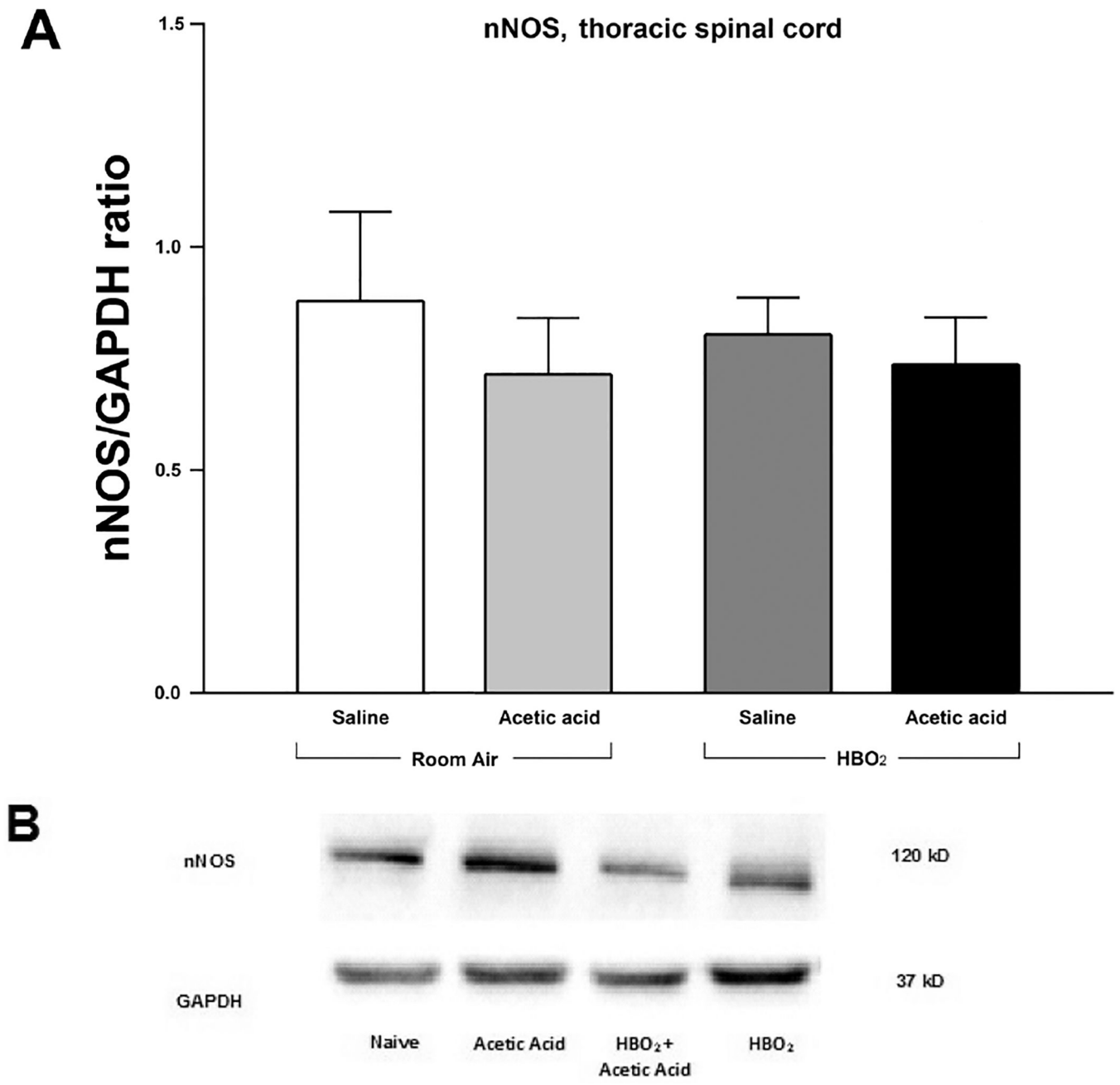

Fig. 8.

(**A**) Effect of glacial acetic acid and HBO_2 treatment on optical density of neuronal nitric oxide synthase in the lumbar section of the spinal cord. No significant differences were found. Each column represents the ratio compared to naïve of nNOS to GAPDH ratios of 6 western blots representing at least 2 separate samples. (**B**) Representative example of western blot of lumbar spinal cord showing expression of nNOS and GAPDH.