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Research article

S-nitrosylation of GAD65 is implicated in decreased GAD activity and oxygen-induced seizures

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HIGHLIGHTS

- Seizures occur in 89% of mice exposed to hyperbaric oxygen (HBO_2) at 4 ATA for 100 min.
- GAD activity decreases, while GABA-T activity remains unaltered in HBO_2.
- HBO_2 increases S-nitrosylation of GAD65, but not of GAD67.
- Results implicate S-nitrosylation of GAD65 for reduced GABA and HBO_2-induced seizures.

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ABSTRACT

Breathing oxygen at partial pressures ≥2.5 atmospheres absolute, which can occur in diving and hyperbaric oxygen (HBO_2) therapy, can rapidly become toxic to the central nervous system (CNS). This neurotoxicity culminates in generalized EEG epileptiform discharges, tonic-clonic convulsions and ultimately death. Increased production of neuronal nitric oxide (NO) has been implicated in eliciting hyperoxic seizures by altering the equilibrium between glutamatergic and GABAergic synaptic transmission. Inhibition of glutamic acid decarboxylase (GAD) activity in HBO_2 promotes this imbalance; however, the mechanisms by which this occurs is unknown. Therefore, we conducted a series of experiments using mice, a species that is highly susceptible to CNS oxygen toxicity, to explore the possibility that NO modulates GABA metabolism. Mice were exposed to 100% oxygen at 4 ATA for various durations, and brain GAD and GABA transaminase (GABA-T) activity, as well as S-nitrosylation of GAD65 and GAD67 were determined. HBO_2 inhibited GAD activity by 50% and this was negatively correlated with S-nitrosylation of GAD65, whereas GABA-T activity and S-nitrosylation of GAD67 were unaltered. These results suggest a new mechanism by which NO alters GABA metabolism, leading to neuroexcitation and seizures in HBO_2.

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1. Introduction

The toxic effects of hyperbaric oxygen (HBO_2) on the central nervous system (CNS) have been recognized for well over a century. In 1944, the Royal Navy documented considerable species differences in susceptibility to CNS O_2 toxicity, such that man and mice were the most susceptible and rats and guinea pigs most resistant [18]. During this time, the U.S. Navy was performing similar experiments and assessing changes in the cortical electroencephalogram (EEG), as well as motor seizure latency in cats exposed to HBO_2 [4,12]. Subsequent studies were carried out in the former U.S.S.R [24], and a consistent pattern begin to unfold: EEG desynchronization (high-frequency waves of low amplitude) preceded hypersynchronous high-amplitude discharges and neuromuscular tonic-clonic convulsions that resembled those observed in patients with epilepsy. Clearly, the EEG patterns and motor seizures induced by HBO_2 were due to neuronal network hyperexcitability, yet the associated neurochemistry remained unknown.

In the 1960s, Wood et al. [27,29] determined that HBO_2 induced neurochemical changes in the brains of mice, hamsters, rats, guinea pigs and rabbits that included a reduction in the concentration of γ-aminobutyric acid (GABA) and the activity of glutamic acid

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decarboxylase (GAD), the rate limiting enzyme that catalyzes the synthesis of GABA from glutamate. Furthermore, the decline in brain GABA was associated with shorted seizure latency, with the critical O_2 partial pressure being 3 atmospheres absolute (ATA) [30]; and intraperitoneal administration of GABA to rats prior to HBO_2 exposure significantly reduced the probability and severity of convulsions [28]. In these investigations, however, brain electrophysiology was not recorded, thus it remained unknown whether GABA decreased prior to or during EEG spikes.

To confirm these observations, our group [8] and others [32] employed EEG monitoring and intracerebral microdialysis during HBO_2 and demonstrated that GABA levels do in fact decrease prior to neuroexcitation. In addition, the ratio of excitatory-to-inhibitory amino acids (glutamate-to-GABA, excitotoxicity index) significantly increased as EEG spikes appeared [8]. Pretreatment with neuronal nitric oxide synthase (nNOS) inhibitor (7-nitroindozol), however, decreased the excitotoxicity index and prevented seizures, implicating NO in CNS O_2 toxicity [8].

The observation that 100% oxygen or NO similarly inhibits human recombinant GAD activity led Davis et al. [5] to hypothesize that NO may modulate GABA production via S-nitrosylation (the covalent attachment of a NO group to a thiol side chain of cysteine, a post-translational modification [14]) of GAD. In order to explore this possibility and expand our own preliminary findings [6], we exposed mice to HBO_2 at 4 ATA and measured the activity of enzymes critical for maintaining the GABA pool, GAD and γ-aminobutyric acid transaminase (GABA-T). GABA-T primarily catalyzes the catabolism of GABA to succinic semialdehyde [22], and irreversible GABA-T inhibition with vigabatrin protects against CNS O_2 toxicity [9,13,23]. The results from these experiments, inhibition of GAD activity with unaltered GABA-T activity, led to subsequent examination of S-nitrosylation of GAD.

2. Methods

2.1. Experimental design and hyperbaric exposures

Awake, freely-moving C57BL/6J wild-type mice (Jackson Laboratories, ME) were exposed to HBO_2 at 4 ATA according to a protocol approved by the Duke University Animal Care and Use Committee. Four sets of experiments were performed: First, mice ($n = 24$) were exposed for 100 min to evaluate the progression of seizure activity. Second, GAD and GABA-T activities were measured in a different group of mice exposed for 30 min ($n = 24$) and compared to mice breathing room air ($n = 10$). Third, GABA-T activity and seizure latency were determined in another group of mice that received an intraperitoneal injection of vehicle (0.9% NaCl, $n = 14$) or vigabatrin (500 mg kg^{-1}, $n = 8$) 3 h prior to a 60-min exposure. Fourth, S-nitrosylation of GAD65 and GAD67 were determined in mice (from experiment two) exposed for 30 min ($n = 11$) and compared to mice breathing room air ($n = 12$). For determining seizure latency, no more than 2 animals were exposed to HBO_2 at one time, whereas for GAD, GABA-T activity, and S-nitrosylation experiments, small groups of 4–8 mice (2–4 per cage) were exposed to HBO_2.

All HBO_2 exposures were conducted by placing the caged mice in a transparent air-filled bag inside a hyperbaric chamber at the Duke Center for Hyperbaric Medicine and Environmental Physiology. The O_2 concentration in the bag was increased to >99%, and the chamber atmosphere was compressed with air to 4 ATA at 0.6 ATA/min. The O_2 and CO_2 concentrations were maintained within the bag at 99.1 ± 1% (SE) and 0.024 ± 0.005%, respectively, due to oxygen flushing during hyperbaric exposure. The chamber temperature was maintained at 25.4 ± 0.3 °C. Prodromal and convulsive signs (heavy grooming, kangaroo-like posture, myoclonus and tonic-clonic convulsions) were recorded.

Immediately following decompression, mice were euthanized with isoflurane and whole brains were isolated and flash frozen in liquid nitrogen and stored at −80 °C for later assessment of S-nitrosylation of GAD65 and GAD67. For determining GAD activity, whole-brains were homogenized in a 10% (wt/vol) solution containing 100 mM $NaPO_4$, 1 mM 2-(2-Aminoethyl) isothiourea dihydrobromide, 0.1% Triton X-100 and 20 μM pyridoxal 5′-phosphate (pH 7.0) and centrifuged at 5000*g* for 30 min at 4 °C. For determining GABA-T activity, whole-brains were homogenized in a 10% (wt/vol) solution containing 10 mM K_2HPO_4, 20% glycerol, 0.13% Triton X-100, 0.1 mM glutathione, 0.1 mM pyridoxal 5′-phosphate and 1 mM EDTA disodium (pH 7.0) and centrifuged at 2000*g* for 20 min at 4 °C. The supernatants were collected and frozen at −80 °C.

2.2. GAD and GABA-T activity

GAD activity was determined by converting glutamate to GABA and subsequently to succinic semialdehyde and then to succinate via GABase (Sigma) and measuring the change in NADPH fluorescence (excitation 340 nm, emission 460 nm) over 30 min [25]. GABA-T activity was determined by converting GABA to succinic semialdehyde and then to succinic acid and measuring the change in NADH absorbance (340 nm) over 10 min [19]. Specific activities were calculated as nmoles of NADPH (GAD) and NADH (GABA-T) $min^{-1} \cdot mg\ protein^{-1}$. Protein concentration was determined using the BCA method.

2.3. S-nitrosylation of GAD65 and GAD67

S-nitrosylation of GAD65 and GAD67 were determined as previously described with modification [16]. Free thiols were blocked with 20 mM methylmethanethiosulfonate (MMTS) and the free nitrosothiols were reduced with 20 mM ascorbic acid to form new thiols that were subsequently biotinylated with 200 μM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (HPDP-Biotin, Pierce). Next, biotinylated proteins were purified by incubating the samples in streptavidin-agarose beads at 4 °C on a rotating wheel overnight. An aliquot of bead supernatant was separated on a 4–20% gel (InVitrogen) and transferred to PVDF membranes (Millipore). Membranes were blocked with 5% BSA in TBST for 1 h and then incubated with anti-GAD65 (1:400, Santa Cruz, sc-5601) and anti-GAD67 (1:200, Santa Cruz, sc-28376) at 4 °C overnight. S-nitrosylated proteins were visualized using horseradish peroxidase conjugated secondary antibodies for GAD65 (goat anti-rabbit, 1:4000, Santa Cruz, sc-2004) and GAD67 (goat anti-mouse, 1:4000, Santa Cruz, sc-2031) with chemiluminescence detection (Santa Cruz).

Images were quantified using ImageJ software (NIH, Bethesda, MD).

2.4. Statistics

A Kaplan-Meier curve was generated to assess seizure latency. An unpaired Students *t*-test was used to determine whether mean group differences were present. A Pearson's correlation coefficient (r) was calculated to determine the relationship between GAD activity and S-nitrosylation of GAD65. Data are presented as means ± standard error of the mean (SEM). Values of $p < 0.05$ were considered statistically significant.

3. Results

Signs of tonic-clonic convulsions were observed in 87.5% of the mice exposed to HBO_2 for 100 min (Fig. 1). Since approximately

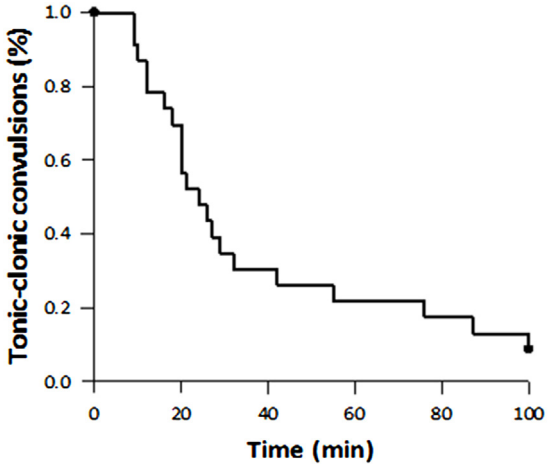

Fig. 1. A Kaplan-Meir plot for tonic-clonic convulsions observed in freely moving mice during HBO_2 exposure at 4 ATA. Mice ($n = 24$) were individually exposed to HBO_2 for 100-min, and tonic-clonic convulsion times were recorded.

50% of the mice displayed seizures at 24 min, the next set of experiments were performed with 30 min exposure times to examine the effects of HBO_2 on GABA metabolism. The results indicate that HBO_2 inhibits GAD activity by 50% ($p = 0.002$) (Fig. 2A), while not-affecting GABA-T activity (Fig. 2B).

To corroborate the role of GABA in oxygen-induced seizures, we administered vigabatrin or vehicle to mice and exposed them to HBO_2 for 60 min. Vigabatrin not only inhibited GABA-T activity by 51% ($p = 0.0006$) (Fig. 3A), but also extended the apparent seizure latency by 25% ($p = 0.003$) (Fig. 3B). Within one hour, 88% of the animals receiving vehicle exhibited tonic-clonic convulsions, while only 25% of mice receiving vigabatrin convulsed.

Since GAD activity was significantly inhibited in mice exposed for 30 min, we selected this time to test whether S-nitrosylation of GAD65 and GAD67 was induced by HBO_2. In these experiments, a 2.5-fold increase in S-nitrosylation of GAD65 was observed with HBO_2 ($p = 0.001$) (Fig. 4A), while there were no significant changes in S-nitrosylation of GAD67 (Fig. 4B). In addition, GAD activity was negatively correlated with S-nitrosylation of GAD65 (Pearson's $r = -0.749$, $p < 0.0001$). Of the 11 mice used for this experiment, 6 exhibited tonic-clonic convulsions, 2 displayed myoclonus, and 3 demonstrated heavy grooming and kangaroo-like posturing.

4. Discussion

The inhibitory effect of HBO_2 on GABA metabolism reported here is consistent with previous reports, indicating a fall in brain GABA content in CNS O_2 toxicity [11,15,26,29,30]. In our studies, GAD activity decrease d and GABA-T activity was largely unaltered in mice exhibiting signs of seizure activity, as previously reported [26,29], in mice exhibiting signs of seizure activity. The combined enzymatic reactions would cause a reduction in GABA synthesis and sustained GABA catabolism, thus a reduced GABA pool that shifts the balance between excitation and inhibition, favoring excitation and predisposing to the development of oxygen-induced seizures. Maintaining GABA levels by providing GABA itself, or preventing its catabolism via GABA-T inhibition with vigabatrin protects against CNS oxygen toxicity as demonstrated here and by others [9,13,23,28]. However, how HBO_2 inhibits GAD activity, leading to a reduction in GABA levels, to our knowledge, had not been explored previously.

It is known that brain nNOS activity increases with elevated inspired oxygen pressure [10], thus the level of NO rises, peaking prior to oxygen-induced seizures [7,21]. NO reacts with superoxide radical ($^{\bullet}O_2^-$) forming peroxynitrite ($ONOO^-$), which is neurotoxic [3]. However, S-nitrosylation is also coupled to the generation of NO by NOS [1], and an increase in protein S-nitrosylation preceding or at the onset of a seizure, is plausible. Indeed GAD65 and GAD67 contain cysteine residues that are likely targets for S-nitrosylation, especially GAD65 [31]. By determining S-nitrosylation of GAD in mice exposed to 4 ATA oxygen for 30 min, an exposure time that corresponded to a 50% reduction in GAD activity, we observed that S-nitrosylation of GAD65 was significantly elevated and was inversely related to GAD activity. These data support the concept that S-nitrosylation of GAD65, acting solely or with one or more other unidentified mechanisms, contributes to the imbalance in glutamatergic and GABAergic neurotransmission that occurs in CNS O_2 toxicity.

The finding that S-nitrosylation of GAD65 increased in HBO_2 at 4 ATA, but that of GAD67 does not, may be due to the respective

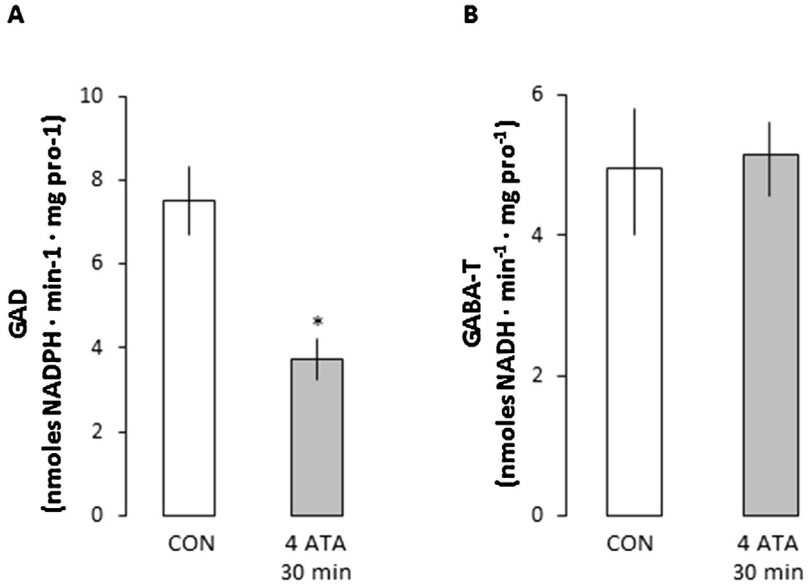

Fig. 2. GAD activity is inhibited by HBO_2. (A) GAD and (B) GABA-T activity were determined in brain homogenates from unexposed control (CON) mice ($n = 10$), and mice exposed to HBO_2 at 4 ATA for 30 min ($n = 24$). *$p < 0.01$ compared to unexposed controls.

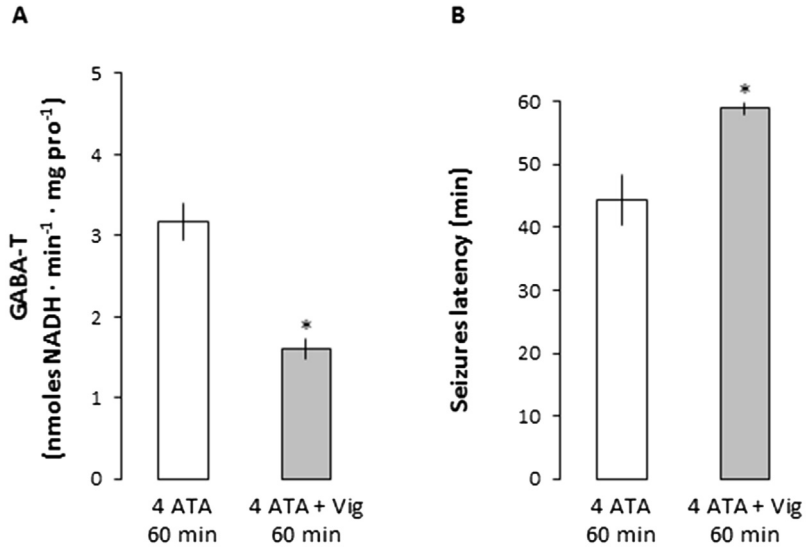

Fig. 3. Vigabatrin inhibits GABA-T activity and extends seizure latency. Vigabatrin (Vig, 500 mg kg body mass^{-1}) or vehicle (0.9% NaCl) was administered intraperitoneally 3 h prior to exposing mice to HBO_2 at 4 ATA for 60 min ($n = 22$). (A) GABA-T activity was determined in brain homogenates immediately following the exposure. (B) Seizure latency, the first appearance of tonic-clonic convulsions, was recorded. *$p < 0.01$ compared to vehicle.

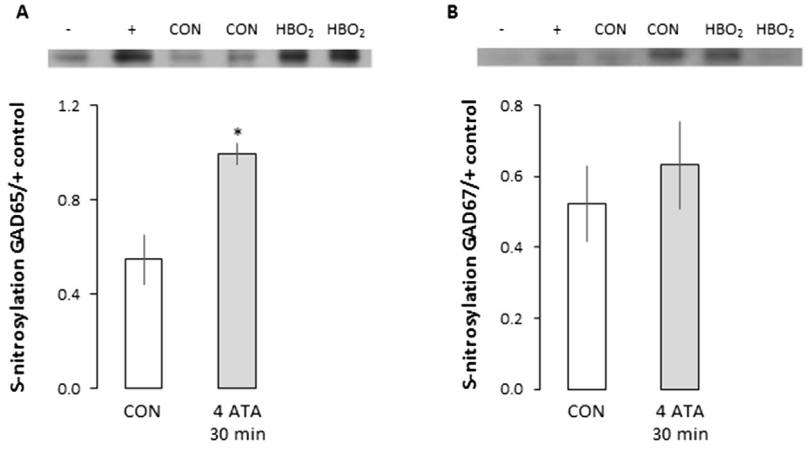

Fig. 4. S-nitrosylation of GAD65 is increased by HBO_2. S-nitrosylation of (A) GAD65 and (B) GAD67 were determined in brain homogenates from unexposed control (CON, $n = 12$), and mice exposed to HBO_2 at 4 ATA for 30 min ($n = 11$). – control (25 mM H_2O_2 was added); + control (1 mM nitrosocysteine and 20 mM ascorbic acid was added). *$p < 0.0001$ compared to control mice.

locations of the isoforms or to the positions of susceptible cysteine moieties in the molecules. Specifically, GAD65 has high probability S-nitrosylation sites at position 30, 45, 73 and 505, whereas GAD67 only at position 32 [31]. In mammalian brain, GAD65 is primarily located in synaptic terminals, whereas GAD67 is widely dispersed throughout the neurons [17]. Mice lacking GAD65 maintain normal GAD67 levels, yet exhibit a shortened seizure latency induced by the GABA-A receptor antagonist picrotoxin [2]. Moreover, GAD65 was reported to catalyze most of the increase in cortical GABA during seizures induced by bicuculline (GABA-A receptor antagonist) [20]. The location of GAD65 would, therefore, serve to provide an immediate source of GABA for hyperpolarizing the post-synaptic neuron. S-nitrosylation of this isoform, however, would oppose this and lead to a reduction in the concentration of GABA and a decrease in inhibitory post synaptic potentials (IPSPs), favoring excitatory post synaptic potentials (EPSPs), culminating in seizures. In spite of S-nitrosylation of GAD, administration of vigabatrin, a so-called suicide inhibitor GABA-T, would slow the rate of decline in the presynaptic GABA pool and delay neuroexcitation. The present data support this concept and provide evidence that a reduction in GABA levels is a significant contributor to HBO_2 induced seizures.

We report the novel finding that S-nitrosylation of GAD65 is increased in brains from mice exposed to 4 ATA of oxygen for 30 min, and inversely related to GAD activity. These results extend our knowledge of the role of NO in oxygen-induced seizures by providing evidence that the well-established reduction in GAD activity can, in part, be attributed to S-nitrosylation of GAD65.

Disclosure

The views expressed are those of HGG and do not reflect the official position of the USUHS or United States Department of Defense.

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