A potential early physiological marker for CNS oxygen toxicity: hyperoxic hyperpnea precedes seizure in unanesthetized rats breathing hyperbaric oxygen

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Pilla R, Landon CS, Dean JB. A potential early physiological marker for CNS oxygen toxicity: hyperoxic hyperpnea precedes seizure in unanesthetized rats breathing hyperbaric oxygen. J Appl Physiol 114: 1009–1020, 2013. First published February 21, 2013; doi:10.1152/japplphysiol.01326.2012.—Hyperbaric oxygen (HBO2) stimulates presump tive central CO2-chemoreceptor neurons, increases minute ventilation (V_min), decreases heart rate (HR) and, if breathed sufficiently long, produces central nervous system oxygen toxicity (CNS-OT; i.e., seizures). The risk of seizures when breathing HBO2 is variable between individuals and its onset is difficult to predict. We have tested the hypothesis that a predictable pattern of cardiorespiratory response precedes an impending seizure when breathing HBO2. To test this hypothesis, 27 adult male Sprague-Dawley rats were implanted with radiotelemetry transmitters to assess diaphragmatic/abdominal electromyogram, electrocardiogram, and electroencephalogram. Seven days after surgery, each rat was placed in a sealed, continuously ventilated animal chamber inside a hyperbaric chamber. Both chambers were pressurized in parallel using poikilocapnic 100% O2 (animal chamber) and air (hyperbaric chamber) to 4, 5, or 6 atmospheres absolute (ATA). Breathing 1 ATA O2 initially decreased V_min and HR (Phase 1 of the compound hyperoxic hyperventilatory response). With continued exposure to normobaric hyperoxia, however, V_min began increasing toward the end of exposure in one-third of the animals tested. Breathing HBO2 induced an early transient increase in V_min (Phase 2) and HR during the chamber pressurization, followed by a second significant increase of V_min ≈8 min prior to seizure (Phase 3). HR, which subsequently decreased during sustained hyperoxia, showed no additional changes prior to seizure. We conclude that hyperoxic hyperpnea (Phase 3 of the compound hyperoxic hyperventilatory response) is a predictor of an impending seizure while breathing poikilocapnic HBO2 at rest in unanesthetized rats.

radiotelemetry; hyperoxia; oxygen toxicity; seizure; diving physiology; submarine medicine; hyperoxic oxygen therapy

BREATHING AN O2-ENRICHED GAS Mixture (e.g., Nitrox) at hyperbaric pressure increases the risk of central nervous system (CNS) oxygen toxicity (CNS-OT) (20). Likewise, breathing pure O2 above 2–3 atmospheres absolute (ATA, where 1 ATA = 760 mmHg) for an extended period (tens of minutes) increases the risk for CNS-OT (2). CNS-OT is manifested as an unpredictable onset of tonic-clonic spasms combined with loss of consciousness. Seizures typically end after the inspired level of oxygen is reduced and thus are not life-threatening per se. The conditions under which CNS-OT can occur, however, make seizures potentially harmful and even life-threatening (e.g., during hyperbaric oxygen therapy for healing a problematic wound or while submerged beneath 30–50 feet of sea water and breathing pure oxygen from a rebreathing apparatus). Currently, the risk of developing CNS-OT is the limiting factor in using hyperbaric oxygen (HBO2) in hyperbaric medicine for wound healing (33); submarine medicine (DISSUB or disabled submarine escape) (7, 40); and closed-circuit technical diving using a rebreathing apparatus for recreational diving, professional diving (oil companies), and military diving in the U.S. Special Operations Forces, which include the Navy SEALs and U.S. Marine Force Reconnaissance units (25).

Previous findings from our laboratory showed that neurons in the caudal solitary complex (cSC) of the dorsal medulla oblongata of rats are among the first neurons under in vitro conditions to be stimulated by normobaric hyperoxia (41), hyperbaric hyperoxia (12, 13, 44), and other chemical oxidants (13, 44, 45). CO2-sensitive neurons in the cSC are believed to function in central CO2 chemoreception and drive ventilation because focal mild acidification of the cSC induces hyperventilation during wakefulness and sleep in rats (47). In addition, protracted exposure to hyperoxia stimulates ventilation in humans and animal models, a paradoxical ventilatory response known as hyperoxic hyperventilation; reviewed by Dean et al. (13). Numerous studies have shown that hyperoxia induces hypocapnia secondarily to hyperventilation (4, 10, 30, 31, 35, 51, 54). The degree to which hypocapnic alkalosis occurs subsequent to hyperoxic hyperventilation, however, has recently been challenged and is currently being debated (21, 22, 28–31).

Regardless, a stimulant effect of 100% oxygen on ventilation—whether it is classified as hyperventilation leading to respiratory alkalosis or hyperpnea without pH change—was recognized in 1947 by Dripps and Comroe (18). It was subsequently shown that carotid body denervation does not abolish hyperoxic hyperventilation or the ensuing decrease in end-tidal PCO2, indicating that the paradoxical hyperventilatory response originates centrally (9, 23, 42). As expected, hyperventilation is greatest during isocapnic hyperoxia, but is reported to be blunted in magnitude and sometimes missed during poikilocapnic hyperoxia because O2-induced hyperventilation secondarily lowers arterial PCO2, and thus the magnitude of O2-induced hyperventilation (3, 4, 51, 54).

The majority of foregoing observations, when considered together, suggest that certain neurons involved in the control of respiration, including CO2-chemosensitive neurons of the cSC, are highly sensitive to oxidative stimuli such as HBO2. Presumably, this is due to the presence of redox- and nitrosative-sensitive enzymes and intermediates (reactive oxygen and nitrogen species) that are located throughout the caudal medulla oblongata, which are activated during protracted exposure to hyperoxic gas mixtures (11). Accordingly, we hypothesize in this study that hyperoxia stimulates ventilation prior to

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onset of CNS-OT when breathing HBO₂, suggesting that it may be a possible physiological indicator or physiomarker of an impending O₂-induced seizure. Our hypothesis is consistent with several anecdotal reports by divers that their diaphragms begin spasmodic contractions prior to onset of HBO₂-induced seizures (60).

To test this hypothesis, we implanted radiotelemetry modules into rats to continuously monitor cardiorespiration and electroencephalographic activity during exposure to hyperbaric hypoxia in unanesthetized and unrestrained male Sprague-Dawley rats. These experiments used poikilocapnic hypoxia to mimic conditions under which CO₂ retention does not occur and, presumably, under which end-tidal Pco₂ decreases secondarily to O₂-induced stimulation of breathing (3, 4, 13, 51, 54). For purposes of this study, however, we will refer to a significant increase in ventilation as hyperoxic hyperpnea, because we did not measure end-tidal PCO₂ or blood gases during exposure to HBO₂. Consequently, we could not determine whether increased depth of breathing, or rate during HBO₂, or a combination of these, caused respiratory alkalosis. Our findings show that hypoxia stimulates ventilation ≤8 min prior to onset of behavioral seizures in an unanesthetized rat. Thus hyperoxic hyperpnea may be a useful physiomarker of an impending CNS-OT seizure in a resting mammal breathing HBO₂.

MATERIALS & METHODS

Animals and Radio Transmitter Implantation

All experiments used male Sprague-Dawley rats (230 – 400 g) obtained from Harlan that were kept on a 12:12-h light:dark cycle (lights-on at 0600 EST; lights-off at 1800 EST). All protocols were previously approved by the University of South Florida Institutional Animal Care and Use Committee (PHS Assurance No. A4100-01; and fully accredited by AAALAC as Program No. 000434), and the Director for Veterinary Affairs, Department of the Navy, Bureau of Medicine and Surgery. Animals were anesthetized initially with 3–5% isoflurane (in O₂) and then maintained on 1–3% isoflurane (in O₂) for the duration of surgery. A subcutaneous injection of the anesthetic Carprofen was given preoperatively, then 5 h after the end of surgery, and finally ~24 h after surgery (5 mg/kg of body wt). The surgical site on the animal was prepared for sterile surgery using standard practices.

In the present study, the 4-ET dual-module transmitter [Data Sciences International (DSI), St. Paul, MN] leads were used to measure two channels of electroencephalogram (EEG), one channel of electrocardiogram (ECG), and one channel of respiratory muscle electromyogram (rmEMG). One pair of leads was implanted at the insertion of the diaphragm muscle at the abdominal wall to record the rmEMG, which was used as the index of breathing (Fig. 1). After placing the rmEMG leads into the respiratory muscles, they were glued in place with a small amount of cyanoacrylate glue. In this configuration, the rmEMG recording was not a pure diaphragmatic signal but represented respiratory activity associated with a mixture of contractions and relaxations made by the diaphragm plus abdominal muscles adjacent to the diaphragm. Presumably, the compound rmEMG recording explains why the rectified, rolling average (ra rmEMG) of each inspiration does not exhibit the typical incrementing pattern (Fig. 1A, middle) as reported typically for pure diaphragmatic EMG recordings and phrenic nerve recordings. Regardless, the ra rmEMG correlated well with the respiratory data collected using the plethysmograph (Fig. 1B).

Another pair of telemetry leads were passed through the abdominal wall and tunneled subcutaneously along the chest using a trocar, and inserted in the pectoral muscle to acquire the ECG recording, using the method described by DSI. The two remaining pairs of leads were passed through the abdominal wall and tunneled subcutaneously along the back and dorsum of the neck using a trocar. Two pairs of wires were exposed through an opening made in the skin beginning on the dorsocaudal aspect of the skull and proceeding rostrally for EEG placement. Two pairs of holes were drilled into the skull using a 2.4-mm drill bit (106 Engraving Cutter, Dremel) at 2 mm bilaterally to Bregma and 2 mm bilaterally to Lambda, as defined by Paxinos and Watson (48). Exposed lead tips were bent 90°, inserted into the drilled hole to make contact with the dura membrane, and held in place with four sterilized stainless steel screws (00-96 × 3/32, 8L0X3905202F; Plastics One, Roanoke, VA). Sterile dental chemically polymerized composite resin (Shade A2, 666320; Integrity) was placed over the polymerized cap and sutured shut.

![Fig. 1. A: a 10-s recording of spontaneous respiration in normobaric air: raw rmEMG, raw compound respiratory muscle electromyogram signal; rect rmEMG, rectified rmEMG signal; ra rmEMG, rolling average of rmEMG signal; Flow, air flow in plethysmograph during breathing; Pleth volume, change in volume in plethysmograph during respiration. B: linear regression (r² = 0.79) derived from the correlation between ra rmEMG (y-axis) and air flow data equivalent to tidal volume collected using the plethysmograph (x-axis, Flow VT). Data show a high correlation between these 2 values, thus confirming the reliability of the modified software to measure tidal volume.](http://jap.physiology.org/doi/fig/10.1152/japplphysiol.01326.2012)
With practice, the entire surgical procedure can be performed in approximately 60–75 min.

Receiver Unit, Video Recording, and Monitoring the Hyperbaric Chamber Environment

Telemetry signals broadcasted from the 4-ET dual-module transmitter were collected by the receiver (model RPC-2; DSI) placed inside the hyperbaric chamber (4 in Fig. 2A). The receiver was placed beneath the chamber housing the animal (3 in Fig. 2A) and cabled to the DSI Data Exchange Matrix box, which was connected to a personal computer for online collection of radiotransmitter biopotentials. Additional nontelemetry signals are collected in real time and interfaced with the DSI system using the data acquisition interface unit (ACQ 7700 Ponemah; DSI), which is located outside the hyperbaric chamber. Nontelemetry signals that were measured included hyperbaric chamber pressure, chamber ambient temperature, and the percent of O2 and CO2 in the breathing gas mixture flowing through the animal chamber (Appendix). Chamber pressure was measured with a pressure transducer and displayed on a digital meter equipped with analog output (DP3002-E; Omega). Chamber temperature was measured with a temperature transducer and displayed on a digital meter equipped with analog output (DP25B-S; Omega). Temperature inside the animal chamber was also monitored with an additional thermocouple. The animal inside the pressure vessel was continuously monitored via a video camera (AXIS 221; Network Camera) connected to the computer. The camera was covered by a light-tight

Fig. 2. A: hyperbaric chamber and animal chamber setup: 1, control panel for regulating chamber pressure and measuring ambient temperature; 2, hyperbaric chamber; 3, animal chamber; 4, telemetry radio-receiver; 5, electrical connector panel; 6, retractable sliding tray for supporting animal chamber and other equipment; 7, main chamber door; 8, darkening housing for video camera. B: schematic representation of air flow in main hyperbaric chamber and parallel O2 (or air) flow in animal chamber.
enclosure to prevent reflections on the porthole window of the hyperbaric chamber that otherwise were detected by the camera and interfered with the video image of the rat (8 in Fig. 2A).

**Acquisition and Analysis Software**

The DSI Ponemah software (P3 Ponemah Physiology Platform, version 4.90) was configured to acquire and record telemetry signals, non-telemetry signals (via the ACQ 7700), and a video signal from each experiment. Analysis modules used with Ponemah 4.90 in this study included raw data (RAW, ver. 4.70), electromyogram (EMG, ver. 4.30), electrocardiogram (ECG, ver.5.10), body temperature (TEMP, ver. 4.70), activity (ACT, ver. 4.70), room barometric pressure (BARO, ver. 4.70), and respiratory muscle EMG (rmEMG, ver. 1.00K). The EMG analysis module purchased with the original system has a minimum temporal resolution of only 10-msec bins. Hence, it lacked the temporal resolution required to calculate tidal volume (VT), respiratory frequency (fresp), and minute ventilation (V˙min) from the rmEMG record during normal to high rates of ventilation in the awake rat. Consequently, the authors contracted DSI technicians to develop a beta version demG (ver. 1.00K) analysis module for reliable measurements of VT, fresp, and V˙min. Temporal resolution of the rmEMG signal was increased down to a minimum value of 0.17-msec bins, thereby significantly increasing the sampling rate allowing measurement of VT, fresp, and V˙min at 4, 5, and 6 ATA (e.g., Figs. 1, 5, and 6).

**Animal Exposure Chamber and Main Hyperbaric Chamber Setup**

The hyperbaric system setup consisted of two chambers that were compressed/decompressed in parallel (Fig. 2A). The main hyperbaric chamber (2 in Fig. 2A; model 20-48-100 hyperbaric research chamber; Reimers System, Lorton, VA) houses the plexiglass animal chamber (3 in Fig. 2A), telemetry receiver (4 in Fig. 2A), and electrical connector panel (5 in Fig. 2A). It is ventilated with air (or nitrogen) and has an internal dimension of 20 × 49 inches when sealed, an internal volume of ~205 liters, and a maximum working pressure of 7.8 ATA. The experiments reported here all used compressed and dried air generated from an oil-less rotary scroll compressor (model DK6086; Powerex, Youngwood, PA) and dryer (SMC, model IDF84E-11N; BCH Mechanical, Largo, FL).

The second main chamber is the plexiglass animal chamber (3 in Fig. 2A; approximately 3-liter volume) made from a recycled whole-body plethysmograph (Diamond Box, model PLY3114; Buxco Electronics, Wilmington, NC), which houses the animal and is ventilated continuously with either air or pure oxygen using a modified hood driver (RSI, Lorton, VA) to regulate gas flow. Thus by keeping the oxygen confined to the animal chamber (contains wires or electrical contacts) and pressurizing the hyperbaric chamber (contains wires and electrical components) in parallel with air or nitrogen, we are able to reduce the risk of a chamber fire (Appendix).

**Normobaric/Hyperbaric Hyperoxia and Dive Profile**

To evaluate the ventilatory sensitivity of each animal to HBO2, rats were individually exposed to hyperbaric hyperoxia three times over 14 days, once every 7 days. The unanesthetized, unrestrained rat was placed in the animal chamber positioned on top of the receiver, which was located inside the hyperbaric chamber. While gathering control data for 10 min, the animal chamber was continuously ventilated with air at normobaric pressure, regulated at a flow rate of ~10 standard cubic feet per hour using the hood driver (Fig. 2B), equivalent to 4.7 liters/min, sufficient to prevent the buildup of exhaled CO2 (not shown). While still at normobaric pressure, the breathing gas was changed to 100% O2 for an additional 15 min (i.e., 1 ATA O2). Keeping the gas supply to each chamber constant (animal chamber, pure O2; and hyperbaric chamber, air), both chambers were compressed in parallel to 4, 5, or 6 ATA (44.1, 58.8, or 75.0 pounds per square-inch gauge pressure, psig) at a compression rate of 0.7 ATA/min, taking care not to exceed a ±1.0 to 1.5 psig pressure differential between the two chambers at all times by manually adjusting the flow rate of compression gas to each chamber. Pressure in the hyperbaric chamber was regulated manually using the main control panel while monitoring its level on the computer screen, and the pressure in the animal chamber was regulated using the hood driver while monitoring its level on a separate pressure gage.

Adiabatic warming of the atmosphere inside the main pressure chamber during compression, and cooling during decompression, were quickly dissipated through the steel walls of the pressure vessel (not shown). This resulted in a modest elevation in air temperature inside the animal chamber during compression to 4, 5, or 6 ATA, which stabilized at ~1 to 1.5°C above control, predive air temperature. Continued exposure to HBO2 activated heat loss responses in rats, as previously reported (6, 50), which lowered core body temperature by ~1°C several minutes prior to seizure by increasing tail skin blood flow (27, and unpublished observations).

**Statistical Analyses**

GraphPad Prism 3 software (version 3.03) was used for all statistical analyses. All values are reported as means ± SE. Data were not normalized to the control values in air or 1 ATA O2; however, because of individual variability in the latency time to seizure (LS) between dives in the same animal and between animals, cardiorespiratory data points were aligned or zeroed to the onset of behavioral seizure (tonic clonic movements of the upper limbs and head) during neurological seizure (increased EEG activity). Data were then analyzed in reverse chronological order until the sample size began decreasing as animals with the shortest-duration LS dropped out of the pooled data (see Figs. 6 and 7B). Thus, regardless of an animal’s individual sensitivity to HBO2 and the duration of its LS, we could determine whether there was a surge or fluctuation in ventilation, or HR, or both, preceding seizure in the majority of animals tested. Likewise, to calculate changes in cardiorespiration for the population before and during onset of compression in HBO2, individual records for each animal were aligned to the onset of compression as the dive commenced, and then analyzed in reverse chronological order (normobaric hyperoxia preceded by air) and chronological order during the next 15–20 min of HBO2 (see Figs. 5 and 7A). The purpose of this second set of analyses was to determine whether onset of poikilocapnic HBO2 breathing initially stimulated breathing that then subsided in part, presumably, due to a hypothesized decrease in arterial CO2 caused by hyperoxic hyperventilation (13). Initially, VT, fresp, and V˙min analyses were performed by averaging the values derived from the raw data every 2 min acquired during three dives per animal. Analyses of VT, fresp, and V˙min prior to diving and seizure involved, first, the statistical comparison of successive 2-min periods preceding (Figs. 5 and 6) and following (Fig. 5) the aligned points. In addition, for Fig. 6, a linear regression of the following time periods was compared statistically for ventilation during the following intervals: 1) 20 to 10 min; and 2) 10 to 0 min before behavioral seizure coincident with neurological seizure. These time periods were selected on the basis of visual inspection of the data, which suggested a surge in ventilation while breathing 4 and 5 ATA O2 that began ~10 min prior to seizure. All statistical comparisons were considered significant at P < 0.05.

**RESULTS**

**Latency Time to Seizure**

In each experiment, LS was calculated starting from the moment at which the chamber had reached the maximum test pressure, or depth, until the onset of behavioral seizures, which always were preceded by seconds to <1 min by neurological seizures (↑ EEG activity). Figure 3 displays the average LS...
Ventilatory Response Prior to Seizure at 4, 5, and 6 ATA

O₂ decreased to 4.8

Susceptibility for seizures was highly variable, especially at 4 and 5 ATA O₂. Nonetheless, as expected, their sensitivity to HBO₂ as measured by their LS value was great, especially at 4 and 5 ATA O₂. The depth of 5 ATA O₂ in the same animal over a 2-wk period. The population trend was for V˙min to decrease in all rats tested while in normobaric hyperoxia (1 ATA O₂) (Fig. 5 right). We call this Phase 1 of the Compound Ventilatory Response to Hyperoxia (described below). Transient hyperoxic hyperpnea at normobaric pressure occurred via a decrease in Vτ and fresp (4 and 5 ATA groups), or Vτ alone (6 ATA group), in one-third of the animals tested. During the final minute of exposure to normobaric hyperoxia, or as barometric pressure increased, conversely, V˙min increased transiently, reaching a maximum level approximately midway through the dive that was similar to the predive level of V˙min. This first, transient relative hyperoxic hyperpneic response during early compression was noted at all three levels of HBO₂. Typically, V˙min increased due to increasing Vτ (4, 5, and 6 ATA; Fig. 5 left) and, in some cases, a transient increase in fresp (4 ATA O₂; Fig. 5 middle). We call this Phase 2 of the Compound Ventilatory Response to Hyperoxia (see below).

As HBO₂ breathing continued, there was a second surge in V˙min that preceded seizure. Hyperoxic hyperpnea begins, on average, ~8 min prior to seizure, on the basis of linear regression analysis. Figure 6 shows the ventilatory data in rows for all animals tested at 4, 5, and 6 ATA. Inspection of vertical columns allows comparison of VT (Fig. 6 left), fresp (Fig. 6 middle), and V˙min (right) responses at each test pressure. Tidal volume increased during the minutes preceding seizure at all three levels of HBO₂ and remained elevated for at least 15 min following the seizure. Respiratory frequency increased transiently prior to seizure at 4 and 5 ATA O₂ but not at 6 ATA O₂. Respiratory frequency decreases either immediately before seizure (5 ATA O₂) or after seizure (4 & 6 ATA). The net effect of these responses was a significant increase in ventilation beginning as early as 8 min prior to behavioral seizure genesis at 4 and 5 ATA O₂ but not at 6 ATA O₂. We call this Phase 3 of the Compound Ventilatory Response to Hyperoxia (see below).

Heart Rate Response Before and During HBO₂ and Prior to Seizure at 5 ATA O₂

ECG data were analyzed off-line using the Ponemah software, and HR values were derived. Figure 7A shows the averaged values (± SE) from 27 dives (9 animals dived 3 times) before and during exposure to 1 and 5 ATA O₂. HR (beats per minute) decreased significantly immediately following exposure to normobaric hyperoxia and continued to decrease throughout the period of normobaric hyperoxia (P < 0.001). Upon compression with pure O₂, HR increased transiently before decreasing prior to reaching 5 ATA (P < 0.001). Continued exposure to HBO₂ caused HR to continue to decrease to a minimum; however, no

Figure 4 shows the results of three dives to a maximum depth of 5 ATA O₂ in the same animal over a 2-wk period. The ventilatory responses to normobaric hyperoxia and HBO₂ were similar between dives. The first 10 min in each panel show ventilation in normobaric air. Increasing inspired O₂ from 21% to 100% decreased ventilation (integrated respiratory muscle electromyogram, int raEMG) throughout most of the 15 min of normobaric hyperoxia. Increasing the level of inspired O₂ from 1 to 5 ATA eventually caused behavioral seizure within 9 to 11 min of achieving maximum depth. Ventilation increased from 1 to 3 min prior to the onset of behavioral seizure. In addition, there was an earlier transient increase in ventilation each time as the dive in HBO₂ commenced (*), which waned and subsided by the time maximum depth (5 ATA) was reached. This suggests that there is an initial transient hyperoxic hyperpneic response when increasing inspired oxygen from 1 to 5 ATA that is followed by a later, second hyperoxic hyperpneic response that precedes behavioral seizure by several minutes.

The averaged data from 71 dives made by 27 animals at three levels of HBO₂ (9 animals/27 dives/test pressure), which are summarized in Figs. 5 and 6, revealed a similar bimodal respiratory response pattern during exposure to 4 and 5 ATA O₂. Like the example shown in Fig. 4, at the start of each dive, the population trend was for V˙min to decrease in all rats tested when normobaric hyperoxia (1 ATA O₂) (Fig. 5 right). We call this Phase 1 of the Compound Ventilatory Response to Hyperoxia (described below). Transient hyperoxic hyperpnea at normobaric pressure occurred via a decrease in Vτ and fresp (4 and 5 ATA groups), or Vτ alone (6 ATA group), in one-third of the animals tested. During the final minute of exposure to normobaric hyperoxia, or as barometric pressure increased, conversely, V˙min increased transiently, reaching a maximum level approximately midway through the dive that was similar to the predive level of V˙min. This first, transient relative hyperoxic hyperpneic response during early compression was noted at all three levels of HBO₂. Typically, V˙min increased due to increasing Vτ (4, 5, and 6 ATA; Fig. 5 left) and, in some cases, a transient increase in fresp (4 ATA O₂; Fig. 5 middle). We call this Phase 2 of the Compound Ventilatory Response to Hyperoxia (see below).

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Figure 3: A: Average latency to seizure (LS; minutes ± SE) for unanesthetized animals exposed to 4, 5, and 6 ATA pure O₂. At 4 ATA, LS was 96.1 ± 10.2 min; at 5 ATA, LS significantly decreased to 29.2 ± 4.4 min; at 6 ATA, LS significantly decreased further to 4.8 ± 0.6 min. B: representation of individual LS values from rats exposed to 4, 5, and 6 ATA O₂. An individual animal’s susceptibility for seizures was highly variable, especially at 4 and 5 ATA O₂. Only the first of 3 dives were used in A and B.
sharp transition toward tachycardia or further bradycardia was noted prior to the onset of CNS-OT (Fig. 7B). Decreasing inspired O₂ increased HR back toward the control level. Figure 7C illustrates the synchronized transient increases in HR (from Fig. 7A) and V<sub>min</sub> (from Fig. 5, 5 ATA) during the onset of compression with pure oxygen.

### DISCUSSION

We studied the effects of a continuum of hyperoxia on cardiorespiration in unanesthetized rats, beginning with normobaric hyperoxia for the following 15 min. Increasing inspired O₂ from 21% to 100% decreased ventilation (integrated respiratory muscle electromyogram, int raEMG) throughout most of the 15 min of normobaric hyperoxia. *Indicates an earlier transient increase in ventilation when the chamber compression started until the time maximum depth (5 ATA) was reached. A similar pattern of ventilation can be observed at 5 ATA for the population when average across all animals tested (Figs. 5 and 6).

Fig. 4. Representative recordings from 3 dives made by the same rat. Data were acquired on day 1 (A; LS = 11 min), day 8 (B; LS = 10 min), and day 15 (C; LS = 9 min) to a maximum depth of 5 ATA O₂. The animal was exposed to normobaric air for the first 10 min, then to normobaric hyperoxia for the following 15 min. Increasing inspired O₂ from 21% to 100% decreased ventilation (integrated respiratory muscle electromyogram, int raEMG) throughout most of the 15 min of normobaric hyperoxia.

The Compound Ventilatory Response to Hyperoxia

**Phase 1:** transient hyperoxic hypoventilation. The compound ventilatory response to hyperoxia exhibits three distinct phases, depending on the O₂ concentration product or dose (level of...
inspired PO₂ × duration of hyperoxia). In the first phase, normobaric hyperoxia inhibits Vₘᵢₙₐᵦ as previously reported (reviewed in Ref. 13). Reduction in Vₘᵢₙₐᵦ while breathing 1 ATA O₂ has historically been attributed to physiological chemodenervation of the peripheral chemoreceptors; that is, breathing 100% O₂ at sea level pressure inhibits the firing rate of the carotid body O₂-chemoreceptors that otherwise are maximally stimulated by hypoxia at normobaric and hypobaric pressures (4, 24, 26). The extent to which the peripheral chemoreceptors are inhibited is debated, however, because not all studies report a hyperoxic hypoventilatory response when breathing normobaric hyperoxia (4, 23, 42, 51, 54). In addition, but less well recognized, is the fact that many investigators have shown that hyperoxic hypoventilation is manifested as a transient response, lasting only during the first 1–3 min of breathing normobaric hyperoxia (13, 18, 61). With continued breathing of normobaric hyperoxia, Vₘᵢₙₐᵦ increases beginning as early as 3–5 min after onset of normobaric hyperoxia (reviewed in Ref. 13). This paradoxical ventilatory response to hyperoxia was reported by Dripps and Comroe (18), who were the first to use normobaric hyperoxia for physiological chemodenervation of the peripheral O₂-chemoreceptors. These authors emphasized, however, that a concurrent stimulatory effect of hyperoxia occurs centrally at normobaric pressure, which limits the duration of hyperoxic hypoventilation and eventually induces hyperoxic hyperventilation. The latter stimulant effect of oxygen that increases ventilation, however, has rarely been recognized by respiratory neurophysiologists (13) despite the fact that hyperoxic hyperventilation at normobaric pressure is well documented in various animal models (23, 42, 61), human infants (52, 56), and human adults (4, 10, 18, 24, 36, 51, 54). In the present study, approximately one-third of the animals tested demonstrated a similar, transient hyperoxic hypoventilatory response that reversed into a relative transient response.
Hyperoxic Hyperpnea Precedes CNS Oxygen Toxicity • Pilla R et al.

Fig. 6. Continuous recordings of averaged data for $V_T$, $f_{\text{resp}}$, and $V_{\text{min}}$ for all rats tested ($n = 27$ rats $\times 3$ dives/rat) during the latter half of HBO$_2$, seizure, and the postseizure period. All data were lined up at the time of behavioral seizure (time = 0). Notice the minute ventilation increases, on average, ~8 min before seizure at 4 and 5 ATA. Refer to the text for additional information. Arrows on bottom traces indicate ~5 min before seizure, which was the average LS value at 6 ATA.

Hyperoxic hyperpnea during the latter half of normobaric hyperoxia.

**Phase 2: transient hyperoxic hyperpnea.** After 15 min of normobaric hyperoxia, the so-called dive was initiated by increasing barometric pressure, which increased the level of inspired O$_2$. This, in turn, caused a transient increase in $V_{\text{min}}$, typically by a sustained increase in $V_T$ that peaked during the first several minutes at maximum pressure (Fig. 5: 4, 5, 6 ATA) and a transient increase in $f_{\text{resp}}$ that peaked before (Fig. 5: 4, 6 ATA) or after reaching maximum pressure (Fig. 5: 5 ATA). A possible explanation for the transient nature of Phase 2 hyperpnea is that sufficient CO$_2$ was blown off during initial exposure to HBO$_2$, which subsequently blunts the ventilatory response to poikilocapnic hyperoxia, as previously reported (3, 4, 51, 54). Hyperventilation, however, could not be confirmed in the present study without concomitant measurements of end-tidal PCO$_2$.

**Phase 3: preseizure hyperoxic hyperpnea.** Protracted breathing of hyperbaric poikilocapnic hyperoxia at 4 and 5 ATA, regardless of the duration of the LS, caused a significant, second increase in $V_{\text{min}}$ that began as early as 8 min before the onset of behavioral seizures. This was the new finding in this study, which also verified our hypothesis. On average, the LS at 4 ATA was 62 min, and at 5 ATA it was 20 min. Hyperoxic hyperpnea prior to seizure, however, was difficult to detect at 6 ATA O$_2$ because behavioral seizures typically occurred within ~5 min of reaching maximum depth. Thus at 6 ATA O$_2$, the early transient hyperpneic response during compression was essentially contiguous with the latter hyperoxic hyperpneic response during the immediate preseizure period.

At 4 and 5 ATA, $V_{\text{min}}$ increased prior to seizure through a sustained increase in $V_T$ until seizure, and a transient increase in $f_{\text{resp}}$ that peaked 2–6 min prior to seizure. Minute ventilation usually peaked at onset of seizure and then immediately recovered due to a reduction in $f_{\text{resp}}$. Tidal volume, in contrast, remained elevated throughout the decompression period and the ensuing 15-min recovery period. Because onset of seizure was the end point of our experiments, we did not study the effects of continued HBO$_2$ exposure on ventilation once seizures occurred. Previous studies have shown, however, that prolonged exposure to HBO$_2$ beyond what evokes CNS oxygen toxicity often evokes irregular breathing and, eventually, respiratory distress followed by respiratory failure and death (2, 58, 59).
We did not examine in this study the underlying mechanisms that produce the compound hyperoxic hyperpneic response. The present study, however, was based in large part on mechanistic data previously reported by this laboratory and others that supported the idea that hyperoxia is a powerful respiratory stimulant (13). We postulate that hyperoxia increases production of reactive O2 and N2 species, which stimulates firing rates of brain stem neurons, including putative CO2-chemoreceptor neurons (44). Additionally, continued exposure to hyperoxia is anticipated to increase PCO2 and [H\(^+\)] secondarily to decreased cerebral blood flow and decreased metabolic CO2 transport (Haldane effect) (15, 57). Longer exposures to hyperoxia may likewise induce pulmonary atelectasis and increased ventilation-perfusion mismatch (5, 53) thereby further contributing to elevated levels of PCO2 and H\(^+\). Increased PCO2 and [H\(^+\)], in turn, will catalyze further production of various reactive species (11) and directly stimulate further ventilation as well (14, 47). The level of hypercapnic acidosis that develops secondarily to hyperoxia and contributes to Phase 2 or Phase 3 hyperoxic ventilation is unclear, however, because any CO2 and protons generated may be offset by O2-induced hyperventilation (4, 10, 30, 31, 35, 51, 54).

The foregoing hypothesis is based on studies of neurons in the cSC. We previously reported that HBO2 and other oxidizing stimuli activated 90% of CO2-excited neurons in the cSC (in vitro), which are believed to function as central CO2 chemoreceptors (14, 44, 47). In addition, neurons in the cSC are also part of the dorsal respiratory group (55) and cardioinhibitory center (34). Neurons in the cSC therefore are capable of influencing cardiorespiration (14).

Cellular CO2 chemosensitivity, however, is distributed throughout the brain stem in other chemosensitive areas (14, 46). Are neurons in other CO2-chemosensitive areas likewise O2-sensitive, and therefore possibly contributing to the phenomena of Phase 2 and Phase 3 hyperoxic hyperpnea? It seems likely that neurons in other CO2-chemosensitive areas would be stimulated by hyperoxia given the wide distribution of the biochemical machinery for redox and nitrosative signaling that exists in respiratory areas of the medulla oblongata (11). Unfortunately, we do not know how widespread hyperoxic-sensitivity is at this time in the brain stem because the study of the effects of normobaric hyperoxia on CNS neurons is largely an unexplored field in respiratory control and neuroscience in general. This is because, essentially, all brain slice (and most cell culture) studies are...
performed under hyperoxic control conditions using 95% O₂, 5% CO₂ in a bicarbonate-buffered medium, as previously reported (12, 43). Interestingly, in the context of the present discussion, a brain slice electrophysiology study using lower levels of control oxygen (40%) reveals that normobaric hyperoxia (90–95% O₂) is an excitatory stimulus in ~50% of cSC neurons tested, including CO₂-sensitive neurons, in medullary slices harvested acutely from neonatal rats (41).

Cardiovascular Responses to Normobaric and Hyperbaric Hyperoxia

Heart rate decreases upon exposure to HBO₂ and remains depressed until seizure onset (19, 32, 37–39). Bradycardia during diving is initiated by hyperbaric pressure alone (37), hyperoxia alone (19), and of course, by hyperbaric hyperoxia (32, 37, 38). HBO₂-induced seizures, in turn, activate powerful cardiovascular reflexes controlled by neurons in the hypothalamus and rostroventrolateral medulla that induce pulmonary hypertension resulting in acute neurogenic pulmonary oxygen toxicity (16, 17). In the present study, we confirmed that HR, likewise, decreased during exposure to, first, normobaric hyperoxia, and secondly, to HBO₂. But whereas \( V_{\text{min}} \) increased significantly in the minutes preceding seizure, we did not observe any particular change in HR that portended an impending HBO₂-induced seizure; that is, the pattern of bradycardia remained unchanged in the minutes preceding seizure. We examined HR only in beats per minute, however, and did not use any other types of analyses that may tease out more subtle changes in autonomic function of the heart, such as the combination of the power spectrum method and principal dynamic mode analysis (1).

The only other change in HR that was noted during compression with pure O₂ occurred when HR began to increase with a time course that was identical to the transient early Phase 2 hyperoxic hyperpneic response (Fig. 7C). Like \( V_{\text{min}} \), HR also decreased prior to reaching maximal depth and then decreased further to a minimum level after ~10 min at maximum pressure. Presumably, the transient stimulation of HR, like \( V_{\text{min}} \), was attributed to the same mechanism postulated to cause the Phase 2 hyperventilatory response discussed above.

Significance of Hypoxic Hyperpnea as a Potential Physiomarker for CNS-OT

Previous studies have reported the phenomenon of hypoxic hyperpnea/hyperventilation at normobaric pressure (4, 24, 42, 51, 54) and hyperbaric pressure (9, 10, 35). This is the first study, however, to confirm that increased ventilation precedes seizure in an awake, unrestrained mammal breathing HBO₂. Historically, the difficulty of calculating safe exposure limits to HBO₂ to maximize its uses both therapeutically (33) and operationally (7, 39, 40), has been the variability exhibited between individuals in their risk for developing CNS-OT. For example, notice the variability in the LS values between rats breathing 4 and 5 ATA of HBO₂ in Fig. 3B. Consequently, the use of HBO₂ clinically and operationally has been limited to the lowest oxygen concentration product that reduces risk in the most sensitive, at-risk individuals in a population. Presumably, exposure time in individuals with greater neurological tolerance to HBO₂ could be extended if an adequate physiomarker was identified to warn of an impending seizure, an indicator that allows adequate time to lower inspired PO₂ and thereby avoid seizure. For example, HBO₂ therapy, which is used for healing problematic wounds, typically uses 90 min total exposure, divided into three 30-min periods interspersed with 10 min of air break, to avoid seizures (2, 33). U.S. Navy divers breathing pure oxygen via a rebreathing apparatus are permitted a maximum of only 10 min bottom time at 50 feet of seawater to avert seizures in all divers (8). In addition, stranded submariners, prior to attempting a rapid, buoyant ascent to the surface of the ocean in a DISSUB emergency, would benefit by denitrogenating (i.e., breathing pure oxygen for 45–60 min) to purge the inert gas nitrogen from their tissues, thus reducing the risk for developing decompression sickness (DCS). Denitrogenation using pure oxygen constitutes a high risk for submariners, however, because the unfortunate event that created the DISSUB emergency has, likewise, compromised the pressure-integrity of the hull of their submerged vessel, exposing them to ambient hyperbaric pressure. Consequently, breathing O₂ at hyperbaric pressure to reduce risk for DCS is now surpassed by an increased risk for CNS-OT seizures during denitrogenation (40). For all these scenarios, establishing a possible physiomarker for predicting onset of seizure in a resting mammal is potentially highly significant for utilizing and extending the clinical and operational applications of HBO₂ in hyperbaric, diving, and submarine medicine.

The present study used poikiloapnic hyperoxia. Consequently, end-tidal PCO₂ was unregulated and could have changed with changes in ventilation (4, 10, 30, 31, 35, 51, 54). It will be important in future studies to employ isocapnic and hypercapnic hyperoxia in addition to poikiloapnic hyperoxia as additional, parallel run experimental protocols because they provide a model for studying the interactions between CO₂ and hyperoxia (i.e., redox/nitrosative stress) on cardiorespiratory control (11). In the context of the present study, the combination of CO₂ and HBO₂ reflects the real-life scenarios that transpire during CO₂ retention when diving using Nitrox or a rebreather (20), during a DISSUB emergency (7), and the use of HBO₂ therapy (33). We postulate that under these conditions that a Phase 3 hyperpneic response that precedes seizure will occur sooner and be even larger under conditions of CO₂ retention (3, 4, 51, 54).

APPENDIX: TROUBLE SHOOTING THE TECHNICAL CHALLENGES OF HYPERBARIC RADIO-TELEMETRY IN RODENTS

Over the course of this study, we encountered numerous technical challenges that required resolution to successfully 1) recycle radiotransmitters for use in multiple animals, and 2) to safely use radiotelemetry under conditions of HBO₂. The following narrative summarizes various problems encountered and how we solved them.

General Considerations—Use of Radio-Telemetry in Acute (1–3 Wk) vs. Chronic (Months) Studies

We initially planned to perform a chronic study in which we used the same animal repeatedly, making a dive in HBO₂ once per week over a 3- to 6-mo period. We discovered, however, that after 2–3 dives ending in seizure, that subsequent dives produce progressively shorter latencies to seizure. Thus there is a history effect, or kindling effect, of prior seizures that increases the risk for CNS-OT (49). If, however, the purpose of the study is to not induce seizures, then radiotelemetry works well for chronic studies lasting several months. The telemetry
module, which provides battery power for the transmitter, has a continuous run-time of 3 mo. By turning the module off between experiments, we have used the same battery for more than 1.5 years, typically making an experimental dive that lasts 1.5–2 h/experiment every 1–2 wk. In addition, the rmEMG leads have never come loose in any of the animals on which surgery was performed, and have remained operational for up to at least 4 mo. The quality of the biopotentials recorded over several months remains unchanged (not shown). The animals also continue to gain weight after implanting the telemetry modules. The average weight at the time of surgery was 393.5 ± 19.1 g (n = 27) and increased as follows: 419.5 ± 16.4 (Week 4; animals used in this study were tested during Week 2 through Week 4), 451.2 ± 14.4 (Week 8), and 484.6 ± 16.9 (Week 16).

Reusing Radiotransmitters in Different Animals

To date, we have implanted the same ten 4-ET telemetry dual modules into 168 rats and made 255 dives in HBO2; that is each 4-ET telemetry module was implanted, on average, into ~17 animals. Not all of these animals were used in the present study, but we mention them here to indicate the hardy nature of these radiotransmitter modules for prolonged use in long-term studies, if required. The manufacturer does not recommend reusing the telemetry modules this many times; however, we have found that it is feasible if care is taken during removal and handling of the telemetry units from the animal following euthanasia, module cleaning and sterilization, and surgical implantation. Sterilization is described by the manufacturer at the following Web address: http://www.datasci.com/information/technotes/392-0027-020.asp.

Typically, resterilization occurs at least 3 days prior to surgery. For the 4-ET dual-module transmitter, we discovered that after plugging and securing the electrical cable from the sensing module into the telemetry module via the set screw that it was necessary to seal the opening through which the cable to the telemetry module passed through using the same silicone compound that was used to seal the set screw in the telemetry module that locks the cable in place (silicone provided by DSI). If this did not occur, body fluids seeped into the telemetry module over time and shorted out the electrical connection with the battery, resulting in loss of all radiotransmitter biopotentials.

In addition, multiple implantations using the same radio transmitter unit will eventually require splicing new lead wire to the original leads. Typically, this becomes necessary due to accidentally cutting the wire(s) during dissection and removal of the transmitter from the animal. Also, progressive shortening of the wire leads occurs each time the transmitter is prepared for implantation into another animal. In the event the biopotential lead becomes damaged or too short, the lead can be repaired using a Standard Multi-color Lead Coupler Kit (276-0146-001; DSI). A Standard Lead Crimp Tool (276-0019-001; DSI) and a Coupler Assembly (370-0104; DSI) will also be required for this repair. The assembly consists of a metal, hollow tube with an internal metal pin that is used to join two leads together. A Lead Coupler Kit manual can be found online on the DSI Web site (http://www.datasci.com/products/accessories/leadcoupler.asp). The final operation for the splicing process consists of applying a thin layer of Adhesive Silicon (370-0137-001; DSI) over the repaired region on the lead. This will decrease the chance that blood/body fluid will enter the lumen of the sheath and cause contamination issues and add a second layer of protection and strength to ensure the leads do not separate.

**Hyperbaric Radiotelemetry**

**Fire safety.** For HBO2 experiments, the primary hyperbaric chamber is compressed with air while the animal chamber is compressed in parallel using pure oxygen (Fig. 2). Thus neither the telemetry receiver nor internal connector panel come in contact with pure oxygen during compression. In our setup, the primary hyperbaric chamber can also be compressed with pure nitrogen instead of air when larger ambient pressures are used. This occurs to avert starting a fire in a chamber filled with HBO2 in the event that an electrical connector shorts out and sparks.

O2 and CO2 profiles in animal chamber during compression/ decompression. To measure %O2 and CO2 flowing through and exiting the animal chamber, a 1/16th-inch OD tubing (high-pressure liquid chromatography stainless steel tubing) penetrated the wall of the pressure vessel and terminated inside at the animal chamber. Outside, the gas line was connected to a flow-meter to regulate gas outflow from the animal chamber into the bottom of an unsealed, 125-ml Erlenmeyer flask into which was inserted the sampling line of the gas analyzer. In this configuration, pressure did not build up inside the flask, which would damage the O2 and CO2 analyzers, yet a reliable sample of gas from the animal chamber was obtained, unaccompanied by room air. The two analog output signals (O2 and CO2) of the gas analyzer were connected to the acquisition interface unit (ACQ 7700 Ponemah).

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**AUTHOR CONTRIBUTIONS**

Author contributions: J.B.D. conception and design of research; R.P. and C.S.L. performed experiments; R.P. analyzed data; R.P. and J.B.D. interpreted results of experiments; R.P. prepared figures; R.P. drafted manuscript; R.P. and J.B.D. edited and revised manuscript; R.P. and J.B.D. approved final version of manuscript.

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