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Effects of Hyperoxia on Brain Tissue Oxygen Tension in Non-Sedated, Non-Anesthetized Arctic Ground Squirrels: An Animal Model of Hyperoxic Stress

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Abstract: Arctic Ground Squirrels (AGS) are classic hibernators known for their tolerance to hypoxia. AGS have been studied as a model of hypoxia with potential as a medical research model. **Problem statement:** Their unique resistance to the stressors of low oxygen led us to hypothesize that AGS might also be adaptable to hyperoxia. **Approach:** This study examined the physiological pattern associated with hyperoxia in response to brain tissue oxygen partial pressure (P_tO_2), brain temperature (T_{brain}), global oxygen consumption (V_{O2}) and respiratory frequency (f_R) using non-sedated and non-anesthetized Arctic Ground Squirrels (AGS) and rats. **Results:** We found that 1) 100% inspired oxygen (F_iO_2) increased the baseline values of brain P_tO_2 significantly in both summer euthermic AGS (24.4 ± 3.6-87.3 ± 3.6 mmHg, n=6) and in rats (18.2 ± 5.2-73.3 ± 5.2 mmHg, n = 3); P_tO_2 was significantly higher in AGS than in rats during hyperoxic exposure; 2) hyperoxic exposure had no effect on brain temperature in either AGS or rats, with the brain temperatures maintaining constancy before, during and after 100% O_2 exposure; 3) systemic metabolic rates increased significantly during hyperoxic exposure in both euthermic AGS and rats; moreover, V_{O2} were significantly lower in AGS than in rats during hyperoxic exposure; 4) the respiratory rates for rats were maintained before, during and after 100% O_2 exposure, while the respiratory responding patterns to hyperoxic exposure changed after exposure in AGS. AGS f_R was significantly lower after hyperoxic exposure than before the exposure. **Conclusion:** These results suggest that hyperoxic ventilation induced P_tO_2 and V_{O2} differences between AGS and rats and led to altered respiratory patterns between these species. AGS and the rat serves as an excellent comparative model for hypoxic and hyperoxic stress studies of the brain.

Key words: Brain temperature, ground squirrels, brain tissue, oxygen partial pressure, respiratory frequency, body mass, temperature correction, barometric pressure, cannula implantation, systemic oxygen

INTRODUCTION

One of the unique polar mammals encountered in an extreme environment is the Arctic ground squirrel (Buck and Barnes, 2000). Its unique metabolism during hibernation is a topic that is just beginning to be understood and has implications for neuroscience (Frerichs, 1999; Ma and Wu, 2008) and disease processes. Hypoxia in the AGS has been the focus of most studies because of its potential to answer questions related to reperfusion injury and neurodegeneration in Alzheimer's Disease, Parkinson's Disease and brain tumors. Hypoxia occurs in relation to certain tumors, promoting angiogenesis, metastasis and resistance to therapy. Pathways involved in hypoxia have profound implications in cancer prognosis and treatment. Hypoxic cells temporarily arrest in the cell cycle, reduce energy consumption and secrete survival and pro-angiogenic factors. On the other hand, hyperoxia has not been studied in this classic polar hibernator.

The inhalation of pure oxygen is often used in medical treatment and activities such as scuba diving and high-altitude flight. However, inhaling pure O_2 even at normal barometric pressure (~760 mmHg) creates unusual stresses within an organism. It is well known that oxygen can have a toxic impact on tissues under conditions of hyperoxia.

The Arctic Ground Squirrel (AGS), a polar hibernating species, is able to endure extreme changes in endogenous O_2 under different natural physiological states. During hibernation, AGS blood is well

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oxygenated with similar blood oxygen partial pressure (P_aO_2) as compared to rat brains (Ma *et al*., 2005), but AGS brain tissue oxygen partial pressure (P_tO_2) is hypoxic (Ma and Wu, 2008). When aroused from hibernation, AGS experiences extreme blood hypoxia (~ 9 mm Hg), but there is no evidence of cellular stress, inflammatory response, neuronal pathology, or oxidative modification in brain following the period of high metabolic demand necessary for arousal (Ma *et al*., 2005). While in the euthermic state, AGS have low P_aO_2 and low hemoglobin oxygen saturation (sO_2) in contrast to non-hibernating rats, indicating a natural mild, chronic hypoxia in AGS blood (Ma *et al*., 2005). However, the P_tO_2 in the euthermic state is higher than in the hibernating state (Ma and Wu, 2008; Ma *et al*., 2009).

Hypoxic blood gases and normal P_tO_2 in euthermic AGS may precondition this species, help them cope with environmental hypoxia and maintain normal brain function.

When euthermic AGS are exposed to 8% O_2 inhalation under non-sedated, non-anesthetized natural conditions, P_tO_2 declines quickly from normal values to very low levels (5 mmHg). P_tO_2 returns to the normal level after hypoxic exposure ends and ambient air inhalation resumes, indicating that AGS brain tolerates hypoxic conditions (Ma *et al*., 2009). When both euthermic AGS and rats are exposed to 100% O_2 inhalation, sO_2 in rats is not affected by 100% O_2 inhalation; however, sO_2 is significantly increased in AGS blood, but remains at a lower level than in rats (Ma *et al*., 2005).

To our knowledge, there have been no investigations demonstrating the response and tolerance differences in P_tO_2 to hyperoxia between hibernating and non-hibernating species. We hypothesize that AGS have a greater tolerance to hyperoxia in the brain than other terrestrial mammals such as the lab rat. This study was designed to examine the physiological mechanism associated with hyperoxia in response to brain tissue oxygen partial pressure (P_tO_2), brain temperature (T_{brain}), systemic oxygen consumption (V_{O2}) and respiratory frequency (f_R) using real time in-vivo measurements of brain tissue oxygenation with "Clark-type" O_2 electrodes in non-sedated and non-anesthetized, free-moving AGS and rats.

MATERIALS AND METHODS

Animals: All procedures were performed in accordance with and approved by University of Alaska Fairbanks Institutional Animal Care and Use (IAUC) guidelines. AGS and Sprague Dawley rats were used for these experiments. Adult AGS of both sexes were trapped during mid-July in the northern foothills of the Brooks Range, Alaska, approximately 40 miles south of the Toolik Field Station (68°38'N, 149°38'W; elevation 809 m) of the University of Alaska Fairbanks (UAF) and transported to Fairbanks under permit from the Alaska Department of Fish and Game. AGS were housed individually at 16-18°C and fed rodent chow, sunflower seeds, fresh carrots and apples ad libitum until mid-September when they were moved to a cold chamber set to an ambient temperature (Ta) of 2°C and 4:20 h light: dark cycle. Sprague Dawley rats (6-7 months of age 5 at time of experiment) were purchased from Simonsen Laboratories (Gilroy, CA) and transported by air to UAF. Rats were housed at 20-21°C, 12:12 h light: dark cycle and fed rodent chow ad libitum.

Surgery for brain guide cannula implantation and abdominal cannulation: The animals used in this research were both male and female. A total of 6 summer euthermic AGS (body mass: 889.2 ± 30.2 g) and 3 rats (body mass: 479.5 ± 20.7 g) were used in stereotaxic surgery for brain guide cannula implantation. The surgeries for AGS and rats were conducted between March and April. Animals fasted at least 12 hours before the surgery. Surgery was performed at room temperature under general anesthesia with isoflurane (Halocarbon Laboratories, River Edge, NJ), induced at 5% and maintained at 1.5-3% mixed with 100% medical grade O_2 at a flow rate of 1.5 L /min. During surgery, the animals' heads were put in a stereotaxic apparatus and their body temperatures were maintained at 35-37°C with a servo-controlled fluid-filled heating pad (Omni Medical Equipment Inc., Cincinnati, O_H).

Under strict aseptic conditions, a skin incision was made midline on the head and the working area was exposed with a retractor. The soft tissues were scraped with a surgical blade and cleaned with sterilized cotton-tipped applicators. After tissue cleaning on the skull, two holes (1.8-mm diameter) to accommodate the guide cannulae were made with a sterilized trephine operated by a battery-driven drill at the coordinates (AP, 13.5 or 14 mm; L, ±3.25 mm; D, -4.0 mm) for AGS and another four holes were made with a steel burr (0.5-mm diameter) near the cannulae holes (two in front of and two in back of the guide cannula holes) on the skull for the foundation of dental cement. After completing the drilling process, four anchor stainless steel bone screws (BAS, West Lafayette, IN) were driven into the skull through the screw holes. Two guide cannulae (CMA11, Acton, MA) were stereotaxically positioned above the right and left striatum (AP, 13.5 or 14 mm; L, ±3.25 mm; D, -4.0 mm), as previously described in detail

(Osborne *et al*., 1999; Zou *et al*., 2002; Ma and Wu, 2008) and were slowly lowered 4 mm from the cortical surface. The surgical procedures for rats were similar to those for AGS. Guide cannulae for rats were stereotaxically positioned above the right and left striatum (A, +0.6 from bregma; L, ±3.5; v, 5.5) with the incisor bar set at -2.4 mm as previously described (Drew *et al*., 1990). Cannulae were secured to the screws with dental cement.

Antibiotics (Baytril; Bayer Corp., Shawnee Mission, KS, dose: 5 mg kg^{-1}) were given twice a day, 1 day before surgery and 2 days after surgery, by subcutaneous injection in the back of the neck. After surgery, animals were allowed to recover completely at room temperature and daily cleanings were performed with 3% betadine for 10-15 days. Following recovery, AGS were housed individually in a cool chamber at 2°C under a light regime of 12:12 hours light: dark; rats were maintained in the same condition as during postoperative recovery.

Calibration for PO_2 electrode and thermocouple and temperature correction for P_tO_2: Calibration of PO_2 electrode and thermocouple. For the measurement of P_tO_2, a PO_2 microelectrode (Model: IPS-020; 200 μm; Inter Medical Co. Ltd, Japan) was calibrated in artificial cerebral spinal fluid (ACSF in mM: NaCl:124; Cl: 2.7; $CaCl_2$:1.2; $MgCl_2$: 0.85; D-Glucose:1.4; $NaHCO_3$:24, pH 7.4) at 37 ^oC equilibrated with air (21% O_2) and N_2 (0% O_2), respectively, before and after each experiment to ensure no obvious change (less than 10%). ACSF solution in a water-circulating double layer glass beaker (15 mL) was thermostatically maintained at a temperature of 37° C by circulating an anti-freezing solution through plastic tubing connected to a circulating bath, which could change the temperature of the circulating fluids to any given temperature from -2 to 40°C. Compressed air containing 21% O_2 was used to set up a standard PO_2 value at a reading of 145-150 mm Hg, depending on the value of atmospheric pressure during calibration and pure compressed nitrogen gas was used to set up a standard PO_2 value as 0 mm Hg on the PO_2 monitor (IMP-201, Inter Medical Co. Ltd, Japan). The needle thermocouples were calibrated at 0 and 40°C with an accuracy of ± 0.1°C in a water circulating bath before gas sterilization.

Temperature correction for PO_2 measurement: Because brain temperature varies at any given time and can also change by exposure to different inhalation gases, P_tO_2 measurement had to be corrected to obtain the real physiological effect. The details of the principle and the procedures of temperature correction for P_tO_2 measurement was described in Ma and Wu, 2008. Briefly, to perform such a temperature correction, the following steps were taken:

- The PO_2 meter was initially calibrated to a PO_2 value of 144-150 mm Hg (the value depended on the local atmospheric pressure during calibration) when the electrode was exposed to ACSF equilibrated with compressed air (21% O_2) at 37°C and to a PO_2 value of zero when ACSF was equilibrated with compressed nitrogen gas (0% O_2) at 37°C
- Under bubbling conditions of 21% O_2 in ACSF solutions ranging in temperature from 0-40°C , the signal outputs of the PO_2 electrode ($P_{O2,\ meas.}$) were measured.
- The actual values of PO_2 ($P_{O2,\ cal.}$), independent of temperature effects, were calculated at each given temperature according to the equation:

$$P_{O2,\ cal.}\ (\text{mm Hg}) = (P_{atm} - P_{H2O}) * FO_2,$$

- where the values of water vapor pressure (P_{H2O}) (Weast, 1972), were appropriate for a given temperature
- These data-$P_{O2,\ meas.}$, $P_{O2,\ cal.}$ and temperature in Celsius (t)-were used to derive a temperature correction factor ($f = A\ e^{-k\ t,}$ where the values of A and k are determined by each electrode calibration) to calculate the actual PO_2 of tissues measured at various temperatures:

$$f = P_{O2,\ cal.} / P_{O2,\ meas.} = A\ e^{-k\ t}$$

"$P_{O2,\ cal.}/ P_{O2,\ meas.}$" were plotted against t at temperatures of 0 to 40° C and the curve function fitted with an Excel program to find the values of A and k. After A and k values were defined, the function, $P_{O2,\ cal.} = (A\ e^{-k\ t})(P_{O2,\ meas.})$, was programmed into the data acquisition program (PowerLab) for online temperature correction of PO_2 measurements.

For each electrode and experiment, the calibration process was repeated as above.

Real-time measurement of P_tO_2 and T_{brain} in the striatum of AGS in conjunction with V_{O2} and f_R: After animals recovered from stereotaxic surgery for

brain guide cannulae implantation, the insertions of the reference electrode, the PO_2 electrode and the micro-thermocouples were conducted under anesthesia with 2-3% isoflurane at room temperature. A sterilized silver-silver chloride electrode (RC1, WPI, Sarasota, FL) was implanted to serve as a reference electrode under dorsal skin on the back through a soft silicone tube; a general anesthetic procedure with isoflurane gas was performed before insertion of the PO_2 electrode and micro-thermocouple. After implantation of the reference electrode, the calibrated and sterilized PO_2 electrode and needle micro-thermocouple for T_{brain} were inserted through the pre-implanted guide cannulae into the left and right striatum, respectively and secured by medical tape. The tips of the PO_2 electrode and needle micro-thermocouple extended about 2 mm beyond the guide cannulae tubes. Buprenophine (0.03 mg kg^{-1}), a pain reliever, was given by subcutaneous injection on the 8 back, with one single dose after insertions of the PO_2 electrode and thermocouple for both AGS and rats. Then for real-time in vivo recording of measurements, the animals were transferred into an experimental chamber with a normal swivel set-up for active animals. The wires connected from PO_2 electrode and reference electrode to PO_2 meter and from thermocouple to thermocouple meter went through a sprint tube and came out the experimental chamber in the middle of the top.

The systemic metabolic rate (oxygen consumption, V_{O2}) of AGS and rats was recorded by indirect calorimetry in a vertical cylindrical-shaped Plexiglas metabolic chamber (I.D. 29 cm, height 32 cm) previously described (Ma and Wu, 2008). Air was drawn into a distribution tube through a vertical cylindrical-shaped Plexiglas metabolic chamber at a flow rate of 3 L/min by a membrane pump and measured by a mass flow meter (model AFSC-10 K, Teldyne Hastings-Raydist, Hampton, VA). A separate pump sampled gas from the distribution tube through a canister, which was filled with molecular sieves and connected in a series with a Nafion drying column. O_2 extraction was measured with an O_2 analyzer (Model: FoxBox 2.0, Sable System International, Inc, Las Vegas, NV), which was calibrated with air. O_2 was calculated with an Excel program on the basis of the O_2 fraction differences between fresh air (before being used by an animal) and expired air (after being used by an animal), flow rate of sampling air, animal body weight and a known respiratory quotient value from previous experiments with AGS (Tøien *et al*, 2001; Ma *et al*, 2005), according to Withers principles of equation 3a: $V_{O2} = V_E*(F_IO_2-F_EO_2)/((1-(1-RQ)*F_IO_2))$, (Withers, 1977). The respiratory quotient value of 0.74 was used for calculation of Sprague Dawley rat RQ cited from the literature (Strohl *et al*., 1997).

The P_tO_2, T_{brain} and V_{O2} were recorded simultaneously 20 minutes after buprenophine injection by a data acquisition program matched with the interface (Power Lab/8 sp, AD Instruments, Inc., Colorado Springs, CO) at a sample rate of 1/min and without further signal filtering. A PO_2 monitor and a thermocouple meter (Sable Systems, TC-1000 thermometer, Henderson, NV) were used as pre-amplifiers and the outputs of those two meters were connected to the interface of the data acquisition program. A Sable interface (model: U12, Henderson, NV) was used as A/D converter for the V_{O2} measurement system. The output signals were then input into the interface of the data acquisition program. All experiments for P_tO_2, T_{brain} and V_{O2} measurement were conducted in the experimental chamber at 18°C. Data in $P_{O2,\ meas.}$ recorded originally by the PO_2 electrode in one channel were calibrated online into corresponding values of $P_{O2,\ cal.}$ without the effects of temperature in another channel.

Respiratory frequency (f_R) was counted manually by direct observation during the entire experimental period with 30-minute intervals beginning 60 min before 100% O_2 exposure and ending 120 minutes after hyperoxic exposure. Counting for f_R was blindly performed by a technician.

Hyperoxic exposure: When the recorded values in P_tO_2 , T_{brain} and V_{O2} were stable for at least 1 hour in fresh air, the air was replaced with 100% O_2. Hyperoxic gas was delivered into the experimental chamber for 3 hours, followed by a return to ambient air. P_tO_2, T_{brain} and V_{O2} were continuously recorded for an additional 2 hours after the return to air. f_R was measured throughout the experimental period. The O_2 percentages in the experimental chamber were monitored with an O_2 analyzer before, during and after 100% O_2 delivery. The mean values of air O_2 fraction inspired by animals before and after hyperoxic exposure in the experimental chamber were 20.91 ± 0.06 (n=9) and 21.20 ± 0.08 (n = 9) mmHg, respectively. The animal inspired O_2 fraction during hyperoxic exposure in the chamber was 100.01± 0.31 mmHg (n = 9).

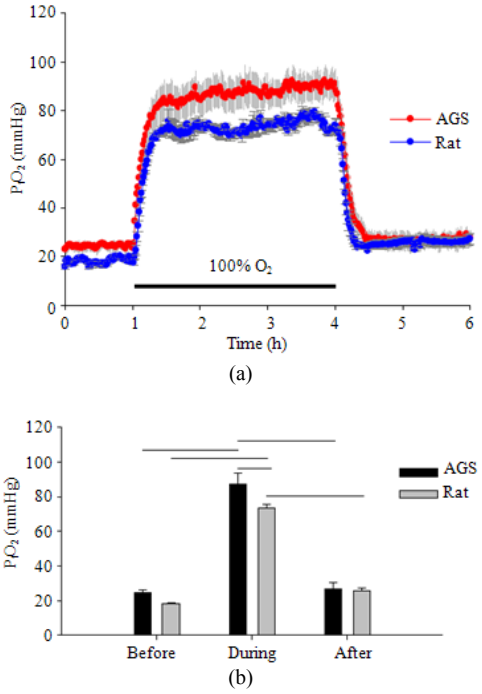

Fig. 1: (a) Effect of 100% O_2 exposure on P_tO_2 (b) Mean P_tO_2 during three experimental conditions

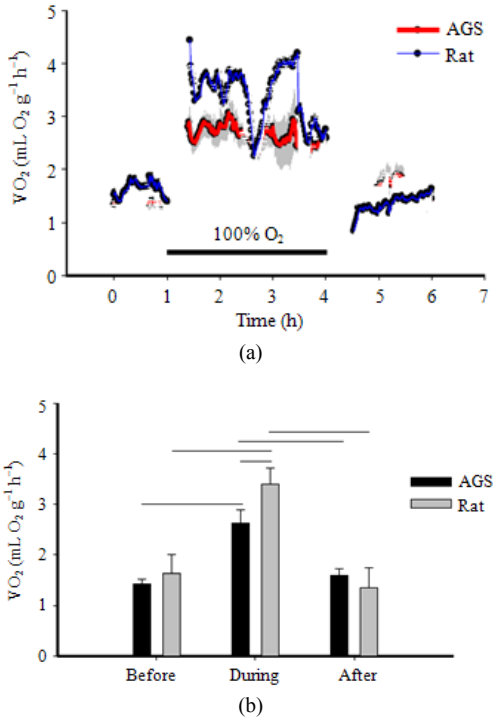

Fig. 2: (a) Effect of 100% O_2 on V_{O2} (b) Mean V_{O2} during three experimental conditions

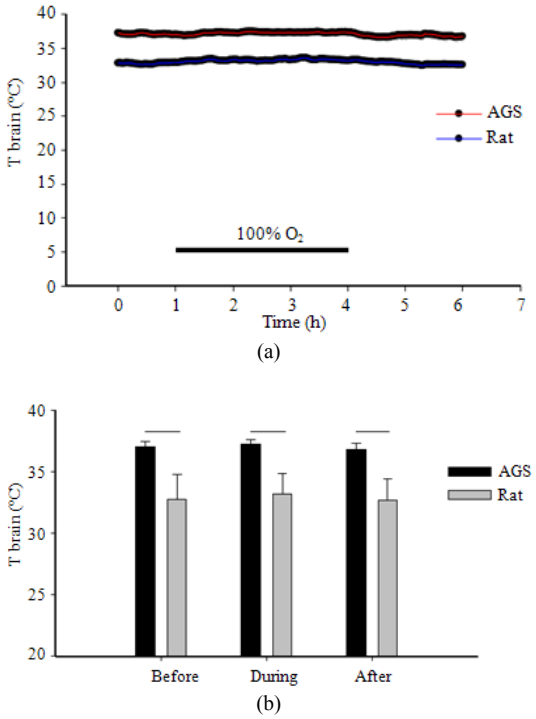

Fig. 3: (a) Effect of 100% O_2 on T_{brain} (b) Mean T_{brain} during three experimental conditions

After P_tO_2, T_{brain}, f_R and V_{O2} recordings were completed, the animals were euthanized with isoflurane under deep anesthetic condition. The brain tissues were immediately frozen in 2-methylbutane at -40°C and kept in a freezer at -80°C. Histologic analysis of the brains using the Cresyl Violet method to identify the PO_2 electrode tip location was performed for each animal, as previously described in detail (Ma and Wu, 2008).

Data analysis: Values in this study are given as mean ± SEM. Data shown in Fig. 1-4 for P_tO_2, V_{O2}, T_{brain} and f_R before, during and after 100% O_2 exposure were averaged to represent the value in each of those three experimental periods. Data for P_tO_2, V_{O2}, T_{brain} and f_R shown in the bar graphs of Fig. 1-3 were analyzed with two-way ANOVA (SigmaStat, version 3.5, Systat Software, Inc. Chicago, IL) using two factors: animal groups (AGS and rats) and 10 experimental conditions (before, during and after 100% O_2 exposure). Significant differences ($p<0.05$) were determined by means of All Pairwise Multiple Comparison Procedure with Holm-Sidak's method. P values <0.05 were taken to represent significant differences. The horizontal lines above the tops of the bars in Fig. 1-4 represent significant differences either between the two groups or between the two experimental treatments.

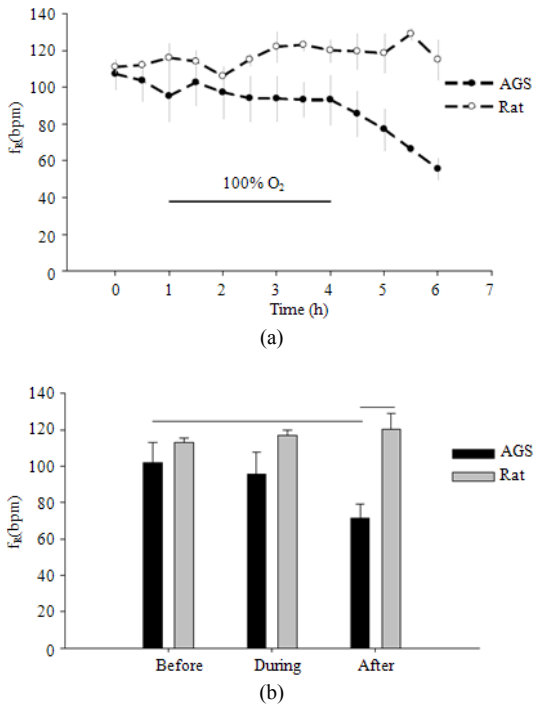

Fig. 4: (a) Effect of 100% O_2 on f_R (b) Mean f_R during three experimental conditions

RESULTS

The response of P_tO_2 to 100% O_2 in the brain: Figure 1, panel A shows the real-time changes in the mean value of P_tO_2 from six AGS and three rats before, during and after 100% O_2 exposure; panel B shows the mean values of P_tO_2 of AGS and rats in these periods. The baseline value of P_tO_2 (24.38 ± 1.91 mm Hg; n = 6) in summer euthermic AGS did not significantly differ from rats (18.19 ± 0.60 mm Hg; n = 3) before 100% O_2 exposure. The P_tO_2 in both AGS and rats increased significantly, (AGS: 87.25 ± 6.17 mm Hg, n = 6; p< 0.001) and (Rats: 73.30 ± 2.24 mm Hg, n = 3; p< 0.001), during 100% O_2 exposure. P_tO_2 increase in AGS was greater than in rats during 100% O_2 exposure (p = 0.038). The P_tO_2 recovered to the levels of (26.91 ± 3.53, n = 6) in AGS and (25.64 ± 1.82 mmHg, n = 3) in rats. For both AGS and rats, there were no significant differences in the groups' P_tO_2 before and after 100% O_2 exposure.

Response of systemic oxygen consumption to 100% O_2: Figure 2, Panel A shows mean real-time changes in V_{O2} before, during and after 100% O_2 exposure. In Panel A, V_{O2} data are missing for the first 30-minute interval after the beginning of 100% O_2 exposure and for the 30 min interval immediately after the end of exposure. Precise V_{O2} values could not be determined during these two transition periods because of the unavailability of a stable O_2 standard in the respiratory system for online calculation of V_{O2}.

Panel B shows the averaged values in V_{O2} during the periods before, during and after 100% O_2 exposure in AGS and rats. Before 100% O_2 exposure the baseline value of V_{O2} of 11 AGS (1.43 ± 0.09 mL O_2 /g hr^{-1}, n = 6) was not different from that in rats (1.64 ± 0.36 mL O_2/g hr^{-1}, n = 3). During hyperoxic exposure, V_{O2} increased significantly in both AGS and rats (P= <0.001), as compared with values before hyperoxic exposure. During the hyperoxic period V_{O2} in AGS (2.64 ± 0.25 mLO_2/g hr^{-1}) was significantly lower than in rats (3.40 ± 0.32 mL O_2/g hr^{-1}) (P = <0.001). During the period after 100% O_2 exposure, V_{O2} recovered in AGS (1.59 ± 0.15 mL O_2/g hr^{-1}) and in rats (1.36 ± 0.39 mLO_2/g hr^{-1}). For both AGS and rats, V_{O2} after 100% O_2 exposure was not different from baseline values before the 100% O_2 exposure.

Response of brain temperature to 100% O_2: Panel A in Fig. 3, demonstrates the real-time changes in brain temperature measured from non-sedated and non-anesthetized euthermic AGS and rats before, during and after exposure to 100% O_2. Panel B shows the average values in brain temperatures before (AGS, 37.06± 0.42°C; Rat, 32.73± 2.03°C), during (AGS, 37.27± 0.38°C; Rat, 33.23± 1.67°C) and after exposure (AGS, 36.80± 0.49°C; Rat, 32.70± 1.76°C) to 100% O_2 in these two species. The brain temperatures maintained constant before, during and after 100% O_2 exposure. Hyperoxic exposure had no effect on brain temperature in either AGS or rats. However, brain temperature is lower in rats than in AGS before, during and after 100% O_2 exposure (p = <0.001).

Response of respiratory frequency to 100% O_2: Panel A in Fig. 4, demonstrates the real-time changes in the respiratory frequency (f_R) measured from non-sedated and non-anesthetized euthermic AGS and rats before, during and after exposure to 100% O_2. Panel B shows the average values in f_R before, during and after exposure to 100% O_2 in these two species. The baseline values of f_R of AGS (102.00 ±10.86 bpm) and rats (113.00 ± 2.33 bpm) showed no significant difference in the present study. The exposure of 100% O_2 did not significantly change f_R in either species during hyperoxic exposure; however, f_R in AGS (71.20 ± 7.86 bpm) was significantly lower than in rats (120.50 ± 8.50 bpm) after 100% O_2 exposure (p = 0.018). In AGS, f_R was also significantly decreased after 100% O_2

exposure (71.20 ± 7.86 bpm) than before the exposure (102.00 ± 9.408 bpm) ($p = 0.032$). Respiratory frequency of AGS declined continuously for two hours after their return to normal 12 air inhalation. Rats maintained a relatively stable level in f_R after cessation of hyperoxic exposure and returning to air.

DISCUSSION

Hyperoxic response and brain tissue oxygenation: High-dose O_2 is routinely used in clinical and non-clinical settings to prevent or treat hypoxemia and tissue hypoxia, as well as controversial treatment of other miscellaneous disorders (Balentine, 1982; Tibbles and Edelsberg, 1996). Little information is available about the precise P_tO_2 response during 100% O_2 exposure under real time in-vivo natural state without anesthetic or sedated effect or whether AGS has a greater P_tO_2 tolerance to hyperoxic exposure than non-hibernating species. The absence of information about the precise P_tO_2 change pattern during 100% O_2 exposure in both hibernating and non-hibernating animals is either caused by anesthetic effect on P_tO_2 or by the lack of an ideal methodology for P_tO_2 measurement of free moving animals or conscious humans. Our study is the first to examine hyperoxic effects on directly measured P_tO_2 in non-sedated and non-anesthetized AGS and rats under 100% O_2.

Our results demonstrate that P_tO_2 in both AGS and rats instantly increase to significantly higher levels during 100% O_2. Similar changes in brain tissue oxygenation during hyperoxic exposures have been reported in non-hibernating animals (Shin *et al*., 2007; Liu *et al*., 2006; Rossi *et al*., 2000; Shinozuka *et al*., 1989) and humans (Hlatky *et al*., 2008; Longhi *et al*., 2002; Macey *et al*., 2007; Tolias *et al*., 2004; Menzel *et al*., 1999) under anesthetic conditions using different O_2 measurement methods. Our P_tO_2 results demonstrate that hibernating species share some of the same response mechanisms as non-hibernating species, like the rat, during hyperoxic exposure. Intriguingly, we found that the response level of P_tO_2 during 100% O_2 inhalation was significantly higher in euthermic AGS than in rats. This result in the response difference of P_tO_2 under hyperoxia offers solid support to our hypothesis that AGS have a greater tolerance to hyperoxia in the brain than other terrestrial mammals such as the lab rat.

When animals or humans are exposed to hyperoxic inhalation, the functions of peripheral chemoreceptors in carotid body are eliminated and those peripheral chemoreceptors become silent (Dean *et al*., 2004; Watt *et al*., 1943; Lahiri *et al*., 2006). Therefore, hyperoxic gas mixtures are routinely used for chemical denervation of peripheral O_2 receptors in in-vivo studies of respiratory control. The response mechanisms under hyperoxic inhalation conditions are commonly considered to be regulated by oxygen-chemosensitive neurons of the central nerve system, which are distributed throughout the brain stem from the thalamus to the medulla (Neubauer and Sunderram, 2004; Dean *et al*., 2003, 2004; Mulkey *et al*., 2001). Therefore, the higher response level of P_tO_2 during 100% O_2 inhalation in AGS compared to rat is accounted by the regulation mechanism controlled by oxygen-chemosensitive neurons of the central nerve system.

P_tO_2 under hyperoxia is maintained by two primary factors, cerebral blood flow (CBF) and P_aO_2 (Hlatky *et al*., 2008). Hyperoxia increases P_aO_2, but decreases CBF in humans (Diringer *et al*., 2007; Hlatky *et al*., 2008). Therefore, the net oxygen delivery to brain during hyperoxia depends on the combined effects of these two factors and other O_2 regulating mechanisms.

Hibernating species have a unique ability to undergo and tolerate dramatic changes in CBF during arousal (Osborne and Hashimoto, 2003, Ma *et al*., 2005). This special CBF regulation mechanism may contribute to a larger increase in P_tO_2 through CBF change during hyperoxic ventilation. As reported in a previous study (Ma *et al*, 2005), hemoglobin-O_2 saturation (sO_2) increases significantly from 85-95% during100% O_2 inhalation in euthermic AGS. This contrasts with rats who maintain a constant sO_2 (~97%) during 100% O_2 exposure. These results demonstrate that euthermic AGS with unsaturated blood hemoglobin-O_2 have a greater capacity than the rats to buffer the hyperoxic exposure. The increase in sO_2 during 100% O_2 inhalation elevates the oxygen content in blood, which further induces a higher brain P_tO_2 in euthermic AGS. Lower systemic oxygen consumption in euthermic AGS compared to rat during 100% O_2 exposure may be another cause for higher P_tO_2 of AGS as indicated in Fig. 2. AGS brain may also have special mechanisms to increase tissue oxygen extraction, as reported for non-hibernating species (Shin *et al*., 2007) and humans (Murthy, 2006).

Despite the benefit of hyperoxia to increase tissue oxygen delivery to brain (Shin *et al*., 2007), hyperoxic ventilation can accentuate the effects of ischemia (Macey *et al*., 2007) and lead to oxidative stress and free radical damage (Liu *et al*., 2006). Paradoxically, hyperoxia results in increased ventilation, leading to hypocapnia, diminished cerebral blood flow and hindered oxygen delivery. Hyperoxic delivery could also induce other systemic changes, including increased plasma insulin and glucagon levels and reduced myocardial contractility and relaxation (Macey *et al*., 2007). To evaluate these side effects of hyperoxic

ventilation on P_tO_2, it is critical to measure P_tO_2 recovery after cessation of hyperoxic ventilation. Our results demonstrated that P_tO_2 in both AGS and rats recovered to the baseline values immediately after returning to ambient air inhalation. However, P_tO_2 of AGS recovered from a significantly higher level to the baseline value after cessation of hyperoxic exposure and return to ambient air. This new finding suggests that AGS brains have a greater tolerance to hyperoxic stress. Their greater ability to recover suggests a more tolerant protection mechanism to overcome the side effects of hyperoxic ventilation. Further histological evidence of the absence of cellular stress and tissue damage is necessary to confirm this physiological result.

Hyperoxic response and metabolic rate for O_2: Under normoxic ventilation (21% O_2 inhalation), changes in the systemic metabolic rate for O_2 (V_{O2}) are temperature dependent in both rats (Dotta and Mortola *et al*., 1992a) and AGS (Buck and Barnes *et al*., 2000). When AGS inhale ambient air, V_{O2} decreases as ambient temperature increase from -16-0°C and remains relatively constant between 0 and 16° C. V_{O2} increases again from 16°C to room temperature (Buck and Barnes, 2000).

With hyperoxic ventilation, the systemic metabolic rate for O_2 increases in non-hibernating animals (Stanek *et al*., 1979; Stock *et al*., 1985; Dotta and Mortola, 1992) and humans (Wilson *et al*., 1975; Mortola *et al*., 1992a, 1992b). In hyperoxia the average values of V_{O2} are above the normoxic values at all ambient temperatures in newborn rats (Dotta and Mortola, 1992). At 20°C, close to the temperature of 18°C used in the present study, hyperoxic values of V_{O2} in newborn rats increased by 73% compared to the values of V_{O2} in normoxia (Dotta and Mortola, 1992).

Diringer *et al*. (2007) found that hyperoxia had no impact on the brain's consumption of oxygen in humans despite their increased P_aO_2 and blood oxygen content. This result suggests that the brain regional metabolic rate does not contribute to increased V_{O2} during hyperoxia in 15 non-hibernating species and humans. However, brain regional metabolic rate in AGS may change during hyperoxia. The brain metabolic response to hyperoxia in hibernating species has not yet been studied.

In this study, we found that V_{O2} during hyperoxic exposure increased and V_{O2} was significantly lower in AGS than in rats. This difference in V_{O2} during hyperoxia was not caused by body mass scaling as discussed in our previous study (Ma *et al*, 2009), where we found that V_{O2} during 8% O_2 exposure was significantly lower in euthermic AGS than in rats. Similar results in V_{O2} changes under opposite inhalation conditions (100 O_2 and 8% O_2) suggest that AGS may have a general mechanism protecting their brains from either hyperoxic or hypoxic stress.

Hyperoxic response and brain temperature: In contrast to the rapid drop of brain temperature during hypoxic inhalation (Ma *et al*., 2009), the brain temperatures in both AGS and rats maintain constancy before, during and after the 100% O_2 inhalation. The ability to maintain brain temperature during normobaric hyperoxic inhalation could account for increased cerebral blood flow (Shin *et al*., 2007), improved cerebral metabolism indicated by biochemical markers (Tolias *et al*., 2004) or maintained cerebral metabolic rate during hyperoxia (Diringer *et al*., 2007). The ability to maintain brain temperature during hyperoxia by AGS and rats demonstrates that these two different species share a protective thermogenic mechanism against hyperoxia.

We previously reported that the baseline value of brain temperature in rats is similar to euthermic AGS. However, in the current study, we observed that rat baseline brain temperature is lower than AGS. In our experience, the core body temperature of euthermic AGS varies between 30-39°C, while rats have a more constant core body temperature between 36-37°C. This greater variation in AGS core body temperature could account for the difference of T_{brain} between the two species. Another contributing factor to the T_{brain} difference could be attributed to depth of the temperature probe in the brain (Zhu *et al*, 2006).

Hyperoxic ventilatory response and f_R: Studies in non-hibernating animals and humans suggest that breathing very high concentrations of oxygen can lead to an increase in ventilation (Becker *et al*, 1996; Dean *et al*., 2004; Forkner *et al*, 2007). Unlike non-hibernating animals or humans, AGS have a very specific ventilation pattern under hypoxic inhalation conditions (Ma *et al*, 2009). AGS f_R decreases with a reduction in ventilation during hypoxic inhalation (8% O_2); rats, however, increase f_R with an elevated ventilation during 8% O_2 exposure. The f_R in rats gradually increases during hyperoxic exposure with 100% O_2 and maintains this increase after exposure. The f_R in AGS show a slight decrease during hyperoxic exposure, followed by a significant decline after hyperoxic exposure ends. The mean value of f_R after 100% O_2 exposure is significantly lower in AGS (71.20 bpm) than in rats (120.50 bpm).

The respiratory control system for non hibernators and humans is particularly sensitive to an increase in inspired O_2. Dean *et al*, 2004 demonstrate that the

ventilation response to hyperoxia is a biphasic response. The first few breaths of hyperoxia (a short lived breath for 1-2 minutes) typically decrease expired minute ventilation (V_E) by inhibiting the peripheral chemoreceptors. The secondary response is a hyperoxic hyperventilation (a dose-dependent increase in tidal volume V_T), which may or may not be accompanied by an increase in f_R. Following "chemical denervation" or "physiological chemodenervation" of the peripheral chemoreceptors, hyperoxic hyperventilation is of central origin. Oxygen has a stimulating effect, which tends to increase respiration and evoke a long lasting facilitation of ventilation. Hyperoxia may alter tonic output for central respiratory drive. The stimulating effect of hyperoxia on respiratory pattern may account for the gradual increase of f_R in rats, the gradually decrease of f_R in AGS and no immediate recovery after hyperoxia in both AGS and rats. Based on this knowledge, we believe that the response difference of f_R between AGS and rat following hyperoxic inhalation may relate to a long lasting facilitation of V_E or changes in tonic output for central respiratory drive.

Animal model for hypoxic and hyperoxic stress: Oxygen pressure in mammalian CNS needs to be maintained at a level which is sufficiently high to ensure undisturbed function of brain cells and sufficiently low to minimize generation of free radicals (Erecinska and Silver, 2001, Al-Hashem, 2010). Excessive oxygen is toxic to mammalian CNS due to over production and accumulation of reactive oxygen species (ROS). Hyperoxia is not a natural existing condition in mammals, but hyperoxia has been a popular model of oxidative stress (Dean *et al*., 2004).

Laboratory reared rats have been used as a model for humanlike ventilatory adaptation to chronic hypoxia (Olson and Dempsey, 1978). Rat is a recognized control animal for hibernating species such as frog (St-Pierre *et al*., 2000), hamster (Fawcett and Lyman, 1954; Nikmanesh *et al*., 1996), bat (Mehrani and Storey, 1997), bear (Fustera *et al*., 2007) and ground squirrel (Fawcett and Lyman, 1954; Popov *et al*., 2007; Ma *et al*., 2005, 2009). Rat is the ususal mammalian representative for human physiology and serves as a proper control for hibernating species.

AGS is a specific hibernating mammalian species that tolerates extreme hypoxic conditions with low cerebral blood flow, low P_aO_2 and unsaturated sO_2 during arousal (Ma *et al*., 2005, 2009). AGS additionally tolerate brain tissue penetration damage without inflammation (Zhou *et al*, 2002). In the current study, we demonstrate that AGS tolerates hyperoxic stress in brain tissue. Because of these tolerances, AGS is an excellent animal model for both hypoxic and hyperoxic stress.

Data from this exploratory methodological study demonstrates the effects of hyperoxic exposure on P_tO_2 in the striatum recorded from non-sedated, non-anesthetized, freely moving hibernating species. Hyperoxic ventilation induces the response differences in P_tO_2 and V_{O2} between AGS and rats and alters the respiratory pattern in AGS following exposure. Since AGS brains have a greater tolerance to hyperoxia than rats, AGS serves as an excellent comparative model for hypoxic and hyperoxic stress.

CONCLUSION

Hyperoxic ventilation induced P_tO_2 and V_{O2} differences between AGS and rats and led to altered respiratory patterns between these species. AGS and the rat serves as an excellent comparative model for hypoxic and hyperoxic stress studies of the brain.

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