Absence of Cellular Stress in Brain Following Hypoxia Induced by Arousal from Hibernation in Arctic Ground Squirrels

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Abstract

Although hypoxia tolerance in heterothermic mammals is well established, it is unclear if the adaptive significance stems from hypoxia or other cellular challenge associated with euthermy, hibernation or arousal. In the present study, blood gases, hemoglobin-oxygen saturation (sO$_2$) and indices of cellular and physiological stress were measured during hibernation, euthermy and following arousal thermogenesis. Results show that P$_a$O$_2$ and sO$_2$ are severely diminished during arousal and HIF 1α accumulates in brain. Despite evidence of hypoxia, neither cellular or oxidative stress, indicated by iNOS levels and oxidative modification of biomolecules was observed during late arousal from hibernation. Compared to rat, hibernating AGS (*Spermophilus parryii*) are well oxygenated with no evidence of cellular stress, inflammatory response, neuronal pathology or oxidative modification following the period of high metabolic demand necessary for arousal. In contrast, euthermic AGS experience mild, chronic hypoxia with low sO$_2$ and accumulation of HIF 1α and iNOS and demonstrate the greatest degree of cellular stress in brain. These results suggest that AGS experience and tolerate endogenous hypoxia during euthermy and arousal.

Key words: Hibernation, torpor, ischemia, stroke, *spermophilus parryii*, hypoxia, reperfusion, inflammation, oxidative stress
Introduction

Hibernation is a unique physiological state of prolonged periods of low body temperature, metabolism, blood flow and other physiological processes that are disrupted by brief periodic arousal episodes when animals rewar and reperfuse metabolically active tissues (7). During arousal thermogenesis blood flow returns to brain and other organs in a reperfusion-like manner at a time of maximal oxygen demand (42, 58). Preservation of neuronal and other cellular morphology during low cerebral blood flow demonstrates that hibernating mammals tolerate pronounced fluctuations in blood flow (16, 61). Physiological and cellular stress experienced during euthermy, hibernation and arousal is less well characterized.

Arterial oxygen tension (\(P_{a}O_2\)) and tissue lactate measurements show that hibernating ground squirrels are well oxygenated (16, 20), sometimes exceeding values in the euthermic state (15). In contrast, oxygen supply may become limiting during arousal thermogenesis. Increases in brain tissue lactate levels during peak oxygen consumption during arousal from hibernation in bats suggest these animals experience oxygen deficiency during arousal and reperfusion (30). However, because tissue lactate was not reported for euthermic bats, it is unclear how brain tissue hypoxia experienced during arousal compares to the euthermic state. Moreover, \(P_{a}O_2\) was not measured to address the relationship between blood and tissue oxygenation during euthermy, hibernation and arousal. To characterize physiological challenges associated with arousal thermogenesis, the present study evaluates blood oxygenation during euthermy, hibernation and arousal along with heart rate, respiratory rate, body temperature and oxygen consumption as signs of metabolic demand.

In addition, brain levels of HIF 1\(\alpha\) were quantified as a marker of tissue hypoxia (49) and, inducible nitric oxide synthase (iNOS) was used as an indicator of inflammation (31, 19).
Finally, although prior studies have demonstrated preservation of gross neuronal morphology during hibernation, no studies have assessed neuronal morphology following arousal from hibernation or more sensitive measures of oxidative stress during euthermy, hibernation or arousal. Thus the present study evaluated gross neuronal morphology as well as oxidative modification of biomolecules in brain to further characterize cellular stress associated with metabolic demands of arousal thermogenesis.

Results show that hibernating AGS (*Spermophilus parryii*) are well oxygenated with no evidence of cellular stress. In contrast, euthermic AGS are mildly and chronically hypoxic. Finally, although arterial oxygen levels (P$_{a}$O$_2$) and hemoglobin- oxygen saturation (sO$_2$) is acutely and severely limited during arousal and HIF 1α accumulates in brain, cellular stress, indicated by iNOS levels, does not increase. Despite evidence of physiological challenge, none of the groups studied showed evidence of histopathology or oxidative modification of biomolecules in brain. Taken together, heterothermic mammals appear to maintain a low level of chronic hypoxia during euthermy and avoid detrimental consequences of endogenous hypoxia, reperfusion and rewarming during arousal thermogenesis.

**Methods:**

**Animals:**

All procedures were performed in accordance with University of Alaska Fairbanks Institutional Animal Care and Use Committee. Arctic ground squirrels (AGS; *Spermophilus parryii*) (group A), 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 of the University of Alaska Fairbanks (68°38’N, 149°38’W; elevation 809 m) and transported to Fairbanks (permit obtained from
Ground squirrels were housed individually at 16-18 °C and fed rodent chow, sunflower seeds, and fresh carrots and apples ad libitum until mid-September when they were moved to a cold chamber set to an ambient temperature \( T_a \) of 2 °C and 4:20 h light:dark cycle. Male Sprague-Dawley rats (6 to 7 months of age at time of experiment) were purchased from Simonsen Laboratories (Gilroy, CA) and transported by air to UAF. Rats were housed in groups of 2 to 4 at 20-21 °C, 12:12 h light:dark cycle and fed rodent chow ad libitum. Groups of AGS were matched for season by using cold-adapted euthermic AGS for comparison with hibernating and aroused AGS. AGS have pronounced circannual rhythms associated with preparation for hibernation including seasonal variation of immune response (52) and antioxidant defense (6). Euthermic, hibernating and aroused animals were also housed at the same ambient temperature in the same environmental chamber to avoid confounding effects of temperature. An ambient temperature of 2 °C is mild for this arctic species where natural burrow temperatures can reach –16 °C during winter and are maintained near 0 °C, cooled by underlying permafrost, in summer (1). AGS housed at or above 18 °C avoid cotton nests and often sprawl on the wire mesh cage bottom to dissipate heat (unpublished observations). Rats, on the other hand, were housed at 20-21 °C, a temperature more appropriate for this species. Winter euthermic AGS consisted of AGS housed at 2 °C in winter (Sept through April) that did not hibernate (most probably due to disturbance since “non-hibernators” will hibernate on other years). Summer euthermic AGS consisted of AGS moved in mid-May to ambient temperature of 16-17 °C and 24:0 h light:dark set to mimic natural lighting conditions. Animals were housed under these conditions for 2 weeks before measuring sO\(_2\). Hibernation is defined as prolonged torpor. Hibernating AGS consisted of animals in prolonged torpor (3-7 days) with respiratory rates less than 6 rpm and body temperature \( T_b \) near ambient temperature (where abdominal \( T_b \) was
monitored via radiotelemetry transmitters (described below) or rectal temperature was monitored with a thermister at time of euthanasia). Late arousal AGS consisted of AGS that had emerged from prolonged torpor in the past several hours and had achieved an abdominal, or rectal body temperature of at least 34 °C. Mid-arousal AGS consisted of AGS emerging from prolonged torpor that had a core body temperature around 10 °C.

**Surgery:**

For blood gas monitoring, rats and AGS were fit with indwelling femoral arterial catheters as described by Tøien et al., (58). Anesthesia was induced with methoxyflurane (Metofane, Schering-Plough Animal Health Corp., Union, NJ) and maintained with halothane (Halocarbon Lab, Riveredge, NJ) 1-3% mixed with 100% medical grade O₂ at a flow rate of 1.5 L/min. During surgery, body temperature was maintained at 35-37 °C with a servo-controlled fluid-filled heating pad (Omni medical equipment Inc., Cincinnati, OH). Under strictly aseptic conditions, catheters were inserted into the femoral artery (Tygon tubing; 0.375 mm i.d., 0.75 mm o.d.; Norton, Akron, OH), externalized in the neck area on the back and sealed. In one group of AGS (group A) abdominal radiotelemetry transmitters (Model WMFH LT 2 cm disc, Mini Mitter Co., Inc., Sunriver, OR) were implanted intraperitoneally via a midline incision through linea alba, and sealed with three layers of sutures. In rats and a second group of AGS (group B) temperature and two lead EKG transmitters (Model TA11CTA-F40, Data Sciences International, St. Paul, MN) were implanted in the peritoneal cavity as above, except that the transmitter was sutured to the abdominal muscle, and the leads were fed subcutaneously and sutured to a sheath overlying pectoralis superficialis in the right ventral shoulder and sartorius of the left inner thigh. Animals were administered Buprenex (Reckitt Benckiser Pharmaceuticals, Inc. Richmond, VA) (0.05 mg/kg, sc) as an analgesic. AGS were allowed at least 1 day post-
operative recovery before being returned to an ambient temperature of 2 \degree C. Rats were returned to ambient temperature of 20-21 \degree C.

**Blood gas sampling and monitoring:**

Blood gases were sampled from AGS during the hibernation season, November through March. An extension was added to the arterial catheter on the day of sampling so that blood could be sampled without touching the animal. Approximately 105 \mu L of blood was sampled 1 to 10 times at 30 min intervals from rats and from AGS during euthermy, hibernation or during arousal from hibernation. Blood gases were measured at 37 \degree C using an I-Stat blood gas analyzer and G3+ or G4+ cartridges (East Windsor, NJ). When multiple samples were collected from rats, euthermic or hibernating AGS results were averaged for a single independent sample. Temperature corrections for blood gases, analyzed at 37 \degree C were performed according to methods of Severinghaus (1966) where oxygen saturation was less than 90\% in euthermic AGS and greater than 90\% in rats and hibernating AGS.

For study of physiological challenge during arousal, animals were divided into two groups. In group A, whole animal oxygen consumption was measured by indirect calorimetry in an open flow system as described previously (58) and respiratory frequency was recorded barometrically with one side of a differential pressure transducer connected to the metabolic chamber. The transducer was connected to a strain gage amplifier and triggering system interfaced to counters on the data acquisition board. Because this method relies on a change in pressure resulting from warming of inhaled gases, respiratory frequencies could not be obtained in hibernating animals before body temperature began to increase. Heart rate was not monitored in this group of animals. Blood levels of lactate and glucose were analyzed with a 2300 STAT glucose/lactate analyzer (Yellow springs Instrument Co., Yellow springs, OH). On the day of
sampling, animals were moved from home cage to recording chamber. After taking a control blood sample, arousal was stimulated by implanting a differential electromyographic (EMG) electrode subcutaneously at the pectoral muscle, and inserting thermocouples into the rectum and cheek pouch. Abdominal temperature ($T_{\text{abd}}$) was recorded with implanted transmitters whose signal was received with RA1000 receivers and BCM100 units connected to a Dataquest III data acquisition system (Mini Mitter Co., Inc., Sunriver, OR). Rectal ($T_{\text{rec}}$) and mouth ($T_{\text{mouth}}$) temperatures were recorded with copper-constantan thermocouples connected to thermocouple amplifiers (AD595, Analog Devices, Inc, Norwood, MA) that interfaced with the data acquisition system.

In a separate group of animals, (group B), heart rate was determined by manual counts from EKG monitored via telemetry and acquired using DataQuest 5 software (Data Sciences International, St Paul, MN). Respiratory rates were counted for 60 sec in hibernating animals and for 10 sec in euthermic animals by visually inspecting the animal prior to blood sampling. In this group, lactate was measured with an I-Stat blood gas analyzer and G4+ cartridges (East Windsor, NJ). Neither oxygen consumption nor glucose was measured. Ambient temperature ($T_a$) during arousal was between 1 and 3°C except in one animal where $T_a$ was 21°C. Data obtained from groups A and B are combined.

Because the hemoglobin-oxygen dissociation curve has not been determined for AGS, partial pressure of oxygen in arterial blood cannot be used to determine percent oxygen saturation ($sO_2$). Therefore, $sO_2$ was measured in winter and summer euthermic AGS and rats using pulse oximetry (Vet/Ox TM 4402L, Sensor Devices, Inc, Waukesha, WI). A rectal probe was inserted under isoflurane anesthesia. Animals were lightly anesthetized with isoflurane mixed with 100% $O_2$ (induced at 5% and maintained at 1% isoflurane) and delivered at a rate of
1.5 L/min. Measurements for sO₂ and heart rate were taken while animals were breathing 100% O₂ and maintained on 1% isoflurane. Anesthesia and oxygen were then discontinued and animals were allowed to breathe room air. Heart rate and sO₂ were monitored until stable and monitoring continued until animals began to move. In a separate group of AGS, probes were inserted in unanesthetized, hibernating AGS. sO₂ and heart rate were recorded within the operating range of the instrument (above 6 °C) and until the animal began to move. Rectal temperature was monitored with a thermister.

Collection of tissues for immunoblotting, histopathology and immunohistochemistry

In separate, non-operated animals, tissue was sampled from 4 groups; rats, euthermic AGS, hibernating AGS and late arousal AGS (late arousal AGS had reached a core Tₘ of at least 34°C; See Animals above for more details). Before initial sampling animals were lightly anesthetized with halothane (5% halothane mixed with O₂ delivered at 1.5 L/min). Rectal temperatures were measured with a thermister. Following decapitation, brains were removed immediately, dissected and frozen in liquid N₂. Frozen tissue samples were stored at -80°C. Time from decapitation to freezing was less than 10 min. The remaining cerebral hemisphere was fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid) and post-fixed overnight. Forebrain was trimmed, paraffin embedded and cut in 7μm sections.

Immunoblotting

Tissue from forebrain of euthermic (n=5), hibernating (n = 5) and late arousal AGS (n = 7) and in some cases rats (n=7) were homogenized in 10 vol. of lysis buffer (50mM Tris-HCl (pH 7.6), 0.02% sodium azide, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 150mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml antipain and 1mM sodium orthovanadate). The protein concentration was determined by BCA assay (Pierce).
Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (40 μg/lane) and electroblotted onto Immobilon-P (Millipore, Bedford, MA) by standard procedures as previously described (Zhu et al. 2001). Transferred blots were incubated sequentially with blocking agent (10% non-fat milk in TBS-Tween), anti-iNOS antibody (polyclonal, made in rabbit; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and affinity-purified goat anti-rabbit immunoglobulin peroxidase conjugate pre-absorbed to eliminate human cross-reactivity. Blots were developed by the ECL technique (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to the manufacturer’s instruction. Blots were stripped in stripping buffer (2% SDS, 62.5 mM Tris-HCl, 100 mM β-mercaptoethanol, pH 6.8) for 30 min at 60 °C and then probed with antibody against actin (1:1000, Chemicon), which is constitutively expressed in neuronal cells. Quantification of the results was performed using a digital image analysis software (KS300, Zeiss). The data obtained were expressed as optical densities.

*Immunoprecipitation:*

Tissue from forebrain of euthermic (n=5), hibernating (n = 5) and late arousal AGS (n = 7) as well as rats (n=7) were homogenized in 10 vol. of lysis buffer (50mM Tris-HCl (pH 7.6), 0.02% sodium azide, 1% NP-40, 150mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml antipain). The protein concentration was determined by BCA assay (Pierce). The homogenate (600 μg) was precleared by incubating with Protein A-agarose (Boehringer Mannheim Corp., Indianapolis, IN) at 4 °C for 2 hours, followed by centrifugation at 10,000 rpm for 10 minutes at 4 °C. 10 μL HIF 1α antibody (monoclonal IgG1 made in mouse, BD Transduction Laboratories) or, as a control, irrelevant Actin antibody (Chemicon) was added to the supernatant and incubated at 4 °C for 4 hours with end-over-end rotation, followed by the addition of Protein A-agarose and incubation overnight. Following centrifugation at 10,000 rpm
for 10 minutes at 4 °C, the supernatant was carefully aspirated and discarded. The pellet was washed 4 times with Lysis Buffer, and the sample boiled for 10 minutes prior to SDS-PAGE. The entire precipitate was loaded in one lane, and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by transfer onto Immobilon-P (Millipore, Bedford, MA) by standard procedures as described earlier. HIF 1α antibody (1:1000, BD Transduction Laboratories) was used for protein detection as described earlier.

**Histopathology and Immunohistochemistry:**

Brain sections were stained with hematoxylin and eosin (H&E) and orange G (OG-6) to assess cellular morphology or processed immunohistochemically using carboxy-methyl lysine (CML; 1:200, polyclonal, made in rabbit) or 4-hydroxy-2-nonenal (HNE-on; 1:100; polyclonal, made in rabbit) to assess oxidative modification of cellular components. Specificity of these antibodies was previously characterized (8, 48). Immunocytochemistry was performed by the peroxidase anti-peroxidase protocol essentially as described previously (41, 64). Briefly, following immersion in xylene, hydration through graded ethanol solutions and elimination of endogenous peroxidase activity by incubation in 3% hydrogen peroxide for 30 min, sections were incubated for 4 min at room temperature in 70% formic acid and then incubated for 30 min at room temperature in 10% normal goat serum (NGS) in Tris-buffered saline (TBS; 50mM Tris-HCl, 150 mM NaCl, pH 7.6) to reduce non-specific binding. After rinsing briefly with 1% NGS/TBS, the sections were sequentially incubated overnight at 4°C with primary antibody. The sections were then incubated in either goat anti-rabbit (ICN, Costa Mesa, CA) or goat anti-mouse (ICN, Costa Mesa, CA) antisera, followed by species-specific peroxidase anti-peroxidase complex (Sternberger Monoclonals Inc. and ICN, Cappel). 3-3’-Diaminobenzidine tetrahydrochrolide (DAB) was used as a chromagen. Hippocampal sections from AD cases,
where studies have found epitopes for both CML and HNE antibodies (8, 48), were always included in each experiment as a positive control.

All chemicals were analytical grade. OG-6 (Harleco brand), Harris hematoxylin stain and Papanicolaou EA-50 (Anapath Co brand) were obtained from StatLab (Lewisville, TX).

**Data Analysis**

Data was analyzed using one or two-way ANOVA followed by pairwise multiple comparisons (Tukey Test) or t-tests when data was normally distributed. In cases where tests of normality failed, data was analyzed using Kruskal-Wallis one way analysis of variance on ranks followed by pairwise multiple comparisons (Dunn’s method) (SigmaStat, SPSS Science Chicago, IL). Regression analysis was performed using Excel (Microsoft Corp.). Data are expressed as group means ± SEM. The criterion for statistical significance was p<0.05.

**Results:**

Blood gas analyses show that $P_aO_2$ from hibernating AGS are significantly higher than normoxic rats. $P_aCO_2$ tended to be lower and pH was significantly higher consistent with suppressed CO$_2$ production (Fig. 1). In contrast, euthermic AGS appear mildly hypoxic, indicated by low $P_aO_2$ and pH and high $P_aCO_2$, all of which are significantly different from hibernating AGS and/or rats (with the exception of $P_aCO_2$) (Fig. 1). $P_aCO_2$ values in euthermic AGS were inversely and significantly correlated with $P_aO_2$ values (p<0.05; $R^2$=0.55).

$P_aO_2$ was decreased significantly during arousal. Fig. 2 shows a sample from one AGS illustrating a drop in $P_aO_2$ at time of peak oxygen consumption. Respiratory frequency increased at time of increased oxygen consumption. Respiratory quotient remained near 0.7 throughout arousal as reported previously (58). Both arterial lactate and glucose concentrations increased during arousal in this animal. During arousal, $P_aCO_2$ increased slightly in this animal although
the increase was not as pronounced as seen in averaged data from a group of animals shown in Fig. 3. Interestingly, $P_a$CO$_2$ did not increase until after the peak in oxygen consumption in this example (Fig. 2). Arterial pH was slightly higher in the hibernating state compared to euthermia at the end of arousal and increased briefly just after maximal oxygen consumption. Mouth temperature increased before abdominal (core) temperature and abdominal temperature increased prior to rectal temperature as reported previously (58). Rapid decline in mouth temperature occurred when the animal became active and dislodged the mouth thermocouple.

Fig. 3 shows means from 7 AGS monitored throughout arousal as well as the group of euthermic AGS from Fig. 1 for comparison. $P_a$O$_2$ consistently dropped to a minimum during arousal. At this time, $P_a$CO$_2$ did not change compared with values in the hibernating state, as would be expected with low CO$_2$ production. However, during mid-arousal, $P_a$CO$_2$ remained low and pH increased at the time of maximal oxygen consumption indicative of hyperventilation and respiratory alkalosis. By the end of the sampling period (late arousal), after abdominal body temperature reached 33.7 °C, $P_a$CO$_2$ and pH, but not $P_a$O$_2$ returned to euthermic levels. Time (mean ± SEM) between minimum $P_a$O$_2$ and end of sampling was 147 ± 11 min (shortest duration of 107 min was in one animal that aroused at $T_a$ of 21 °C). Average duration $P_a$O$_2$ remained below 35 mmHg was 207 ± 23 min with only one of 7 AGS reaching $P_a$O$_2$ above 35 mmHg by the end of sampling. The change in respiratory frequency during these two stages of arousal was not statistically significant (Fig. 3) in contrast to results from a single animal shown in Fig. 2. This discrepancy may be because increases in respiratory volume (not measured) contributed to overall increase in respiration. Similarly, no significant difference in arterial lactate was observed in group means although arterial lactate increased in one out of 7 AGS
during arousal (Fig. 2). Lactate at time of minimal $P_aO_2$ did not correlate with $P_aO_2$ or $P_aCO_2$ ($p>0.05$). Heart rate and oxygen consumption peaked when $P_aO_2$ was at a minimum (Fig. 3).

Consistent with low $P_aO_2$, hemoglobin-oxygen saturation ($sO_2$) was significantly lower in euthermic AGS than in rat when animals were breathing 100% $O_2$ or room air (21% $O_2$), ($p<0.001$, Two Way ANOVA, for main effect of gas, species and gas x species interaction) (Fig. 4). There was a significant decrease in $sO_2$ in euthermic AGS ($p<0.001$) when animals were switched from 100% $O_2$ to room air. Rats breathing 100% $O_2$ or room air maintained $sO_2$ at 97% or greater and $sO_2$ did not decrease significantly when animals were switched from 100% $O_2$ to room air ($p=0.376$). The $sO_2$ in winter and summer euthermic AGS breathing room air was only 83% (n=9) and 85% (n=10), and there was no statistically significant difference between these two groups of animals ($p=0.302$, t-test). In both winter and summer AGS breathing room air $sO_2$ was significantly lower than rat ($p<0.001$, t-test).

Heart rate, recorded at the same time as $sO_2$, was not affected by light anesthesia or recovery from anesthesia in either species. The mean heart rate in winter euthermic AGS breathing 100% $O_2$ and room air was 261 ± 16 and 233 ± 17 beats per minute (bpm) (n=9, body weight: 731 ± 40 g). The mean heart rate in rats breathing 100% $O_2$ and room air was 347 ± 3 and 344 ± 3 bpm (n=8, body weight: 366 ± 8 g). There was no significant difference in heart rate between winter and summer euthermic AGS under any condition ($p=0.406$, ANOVA).

During arousal from hibernation, the mean minimum $sO_2$ in AGS was 57 ± 10% (n=5, body weight: 803 ± 45g) (Fig. 4). Rectal $T_b$ was 10 ± 2 °C, and heart rate was 42 ± 6 bpm at the time minimum $sO_2$ was recorded. There was no significant difference in body weight among winter, summer and mid-arousal AGS in which $sO_2$ was measured ($p=0.406$, ANOVA).
To begin to relate arterial blood gas levels to tissue oxygenation, protein levels of HIF 1α were measured in forebrain as an indicator of tissue oxygenation. Consistent with low $P_aO_2$ in euthermic and late arousal AGS, HIF 1α was significantly higher in these two groups of animals when compared with hibernating AGS or normoxic rat (Fig. 5).

To explore the intracellular consequence of arousal reperfusion/thermogenesis in brain, we measured the levels of inducible NOS (iNOS) as shown in Fig. 6. Indeed, iNOS expression is hardly detectable in hibernating animals while dramatically induced (2.5 fold increase) in euthermic animals. However, the expression of iNOS is not induced in late arousal AGS brain despite reperfusion.

None of the histopathological parameters examined showed evidence of pathology or increased intraneuronal oxidative modification as indicated by CML or HNE immunostaining, markers of oxidative damage to proteins and lipids respectively (Fig. 7). AD tissue, run in parallel as a positive control, showed dark immunoreactive product with both CML and HNE antibodies. Further, there was no overt neuronal pathology observed in H&E stained sections from late arousal animals (not shown).
Discussion:

Hibernation provides significant neuroprotection (63, 14) that is beginning to be appreciated for multifaceted mechanisms that may be linked to other models of ischemia and hypoxia tolerance (55, 12). Still, little is understood regarding physiological and cellular stress in heterothermic mammals. We report for the first time, that euthermic AGS show evidence of low respiratory drive and associated mild, chronic hypoxia with low hemoglobin-oxygen saturation and accumulation of HIF 1α and iNOS. Moreover, AGS show signs of hypoxia following arousal, without evidence of cellular stress.

Hypoxia during euthermy

P₃O₂ values of less than 80 mmHg have been reported in euthermic animals of other heterothermic species (40, 5, 15, 29), although such low P₃O₂ levels have not been observed consistently (56, 16). Low P₃O₂, high P₃CO₂ and low pH has been observed regularly in euthermic AGS in our laboratory over the past several years. Sampling from unanesthetized, freely moving animals via cannula extensions avoided stress effects on blood gases. Moreover, euthermic blood gas values could not be explained by obesity. While AGS are seasonal fatteners, blood was sampled during the winter season when AGS are typically the leanest. Furthermore, no difference in body weight was noted between hibernating and euthermic animals. Low sO₂ in summer and winter AGS (83-85%), further supports the interpretation that this species sustains, mild, chronic hypoxia. Rats, like humans and most other mammals, maintain sO₂ of 95% or greater, and sO₂ of less than 95% is an indication for supportive therapy (45, 18, 28). The sO₂ results reported here suggest that the oxygen-hemoglobin dissociation curve is not left shifted in AGS as it is in two other heterothermic species. In Golden Mantled and Thirteen-lined ground squirrels a P₃O₂ of 50 mmHg corresponds to an O₂ saturation of about
90 percent (40, 34). Alternatively, if the curve is left-shifted in AGS, other factors such as high 
P_aCO_2 and low pH observed in AGS may contribute to low sO_2 in these euthermic animals.

To better address tissue hypoxia, we measured HIF 1α levels in brain. It is well known that HIF 1α accumulates during hypoxia (25, 49). Exposure to 14 percent oxygen (equivalent to 
P_aO_2 of 60mmHg in rat) is sufficient to increase brain levels of HIF 1α. Here, we measured for the first time, HIF 1α levels in brain of heterothermic mammals as an indicator of tissue O_2 deficiency and found that euthermic AGS have higher HIF 1α levels than hibernating AGS. High levels of HIF 1α can be interpreted as low brain tissue oxygen. It is of interest that a recent report showed that elevated HIF1α levels were correlated with activation of JNK and ERK (65). In addition to evidence of tissue hypoxia, elevated iNOS, a marker of cellular stress (44, 23, 24) suggests cellular stress pathways are activated in euthermic AGS. The iNOS isoform is also necessary for some forms of preconditioning (9, 26, 51, 59). Cellular stress, indicated by p38 activation, was shown previously to be greater in summer euthermic AGS housed at 20 °C than in winter euthermic AGS housed at 2 °C suggesting seasonal differences in cellular stress (65). Based on the present results, a difference in sO_2 can not explain the greater p38 activation in summer euthermic AGS brain since sO_2 was not different between winter and summer euthermic AGS.

**Normoxia during hibernation**

P_aO_2 and P_aCO_2 in hibernating AGS are similar to other reports (15, 40) and consistent with pronounced metabolic suppression and decreased CO_2 production during hibernation (4). Elevated blood pH is consistent with one reported result from winter euthermy (56), but differs from most other studies where pH of hibernating and euthermic ground squirrels do not differ (16, 15, 27, 40, 29). Persistently high blood pH in hibernating AGS furthermore contrasts with
acid/base regulation in homeothermic mammals. A similar decrease in $P_aCO_2$ and increase in pH in humans, for example, resulting from respiratory alkalosis is normally compensated for by decreased bicarbonate ion reabsorption by the kidneys and a subsequent normalization of pH. Elevated pH in hibernating AGS may persist because renal blood flow, filtration and regulatory control decrease along with most other physiological processes during hibernation (54). Moreover, blood in hibernating AGS is not as alkaline as it appears because neutral pH increases at colder temperatures. Hemoglobin affinity for $H^+$ increases at colder temperature causing pH to change approximately 0.015 per degree Celsius, and this is corrected for in our reported pH values as described by Severinghaus (50). In addition, because the dissociation constant of water decreases with decreasing temperature, as temperature drops, neutral pH shifts to higher value. For example, while neutral pH is 7.0 at 25°C, it is 7.3 at 5°C (60). Thus, the higher pH in the hibernator is not more alkaline because of the effect of temperature on the water dissociation constant. Comparable brain levels of HIF-1α in hibernating AGS and rats suggest brain tissue is well oxygenated during hibernation.

**Hypoxia during arousal**

The blood gas and sO$_2$ results reported in the present study show, for the first time, that AGS experience temporary, albeit severe endogenous global hypoxemia during arousal that coincides with a peak in metabolic demand. Thus, during arousal thermogenesis, when cerebral blood flow surges at the period of high metabolic demand (42) and animals reperfuse metabolically active tissues, oxygen delivery fails to keep pace with demand producing a period of severe hypoxemia with $P_aO_2$ falling to 9 mmHg and lasting 4 h and sO$_2$ reaching a minimum of 57%. Although unloading of O$_2$ should be enhanced during warming and transition to
euthermy (34), O\textsubscript{2} supply does not appear to keep pace with metabolic demand and this leads to a period of severe hypoxia during arousal.

Elevated levels of HIF 1\(\alpha\) in brain during late arousal is consistent with brain tissue hypoxia. Rat brain cortex rapidly accumulates HIF 1\(\alpha\) during the onset of exposure to 10% O\textsubscript{2} (10) suggesting that the time course of tissue sampling is sufficient to observe an increase in HIF 1\(\alpha\) caused by hypoxia during arousal.

Why a consistent increase in arterial lactate, associated with hypoxia during arousal, was not observed in the present study may relate to decreased glucose availability and the lack of carbohydrate metabolism during hibernation and arousal. Concentrations of glucose in plasma decrease by about half (from 10 to 5mM) within the first day of torpor in AGS (43) and although gluconeogenesis replenishes some glycogen stores during arousal (17), glycogen pools are largely depleted (35). Moreover, the activity of glycogen synthase is decreased during hibernation (21). Indeed, ground squirrels are known to rely primarily on fatty acid oxidation during hibernation and arousal indicated by a respiratory quotient of 0.7 throughout hibernation and arousal (58, 11). One isolated case of an increase in plasma lactate in the present study as well as prior reports of increased plasma lactate during arousal in AGS (17) suggest individual variation may account for differences in results.

Enhanced ATP demand/coupling during hibernation and arousal is another possible explanation for unexpectedly stable arterial blood lactate levels during arousal (36) and is consistent with evidence showing that energy balance is maintained during arousal from hibernation (32). In contrast to the absence of a reliable lactate response during arousal, exposure of euthermic AGS to 8% O\textsubscript{2} increases arterial blood lactate concentrations 3 fold or more (Ma YL, Cozad KD, Rivera PM, Zhao, HW, Drew KL, unpublished observations).
Attenuated cellular stress during arousal

iNOS is barely detectable during late arousal suggesting attenuated activation of intracellular stress signaling pathways. Previously we reported an absence of inflammatory response in hibernating AGS, and concluded that immune modulation contributed to pronounced neuroprotection (63). It is notable that iNOS expression is significantly associated with p38 activity in hibernating animals (65). Therefore, in aroused animals, suppressed p38 activation and iNOS levels downstream to p38 may help to attenuate the inflammatory response during arousal, when blood flow returns in a reperfusion-like manner.

Alternatively, iNOS appearance might be delayed in arousal such that expression was not maximal at the time tissue was sampled. Cold body temperature, evident at the time of most severe hypoxia, may be one mechanism of attenuating the inflammatory response. Mild, hypothermia (33°C), applied during the ischemic period, attenuates increases in iNOS and reactive nitrogen production. Such an effect may be an important mechanism of hypothermia-induced neuroprotection (19).

Histopathology and oxidative modification

Reperfusion after ischemia provides oxygen as a substrate for numerous enzyme oxidation reactions that produce free radicals to such an extent that antioxidant systems are overwhelmed. This oxidative stress results in oxidative damage, including lipid peroxidation, protein oxidation and DNA damage, which can lead to cell death (62). Multiple lines of evidence demonstrate that reactive oxygen species (ROS) are generated during re-perfusion, hypoxia and re-oxygenation and cause oxidative damage to important cellular components, that contribute to cell death (2, 22, 3, 57, 53). We therefore examined brain tissues from euthermic, hibernating and late arousal AGS for cellular damage and oxidative modification. Prior studies
show that lipid peroxidation and protein oxidation occurs mainly during the period of reperfusion (39) suggesting that oxidative stress incurred during arousal would be evident within the time course studied. CML is a rapidly formed, stable product of both lipid peroxidation and glycation processes (46) and HNE, is a stable marker of lipid peroxidation (47). Absence of oxidative modification in brain is consistent with our previous studies showing that reduced glutathione and ascorbate are either maintained in brain throughout hibernation and arousal or increased slightly during late arousal (13, 58, 33).

Most studies show that hibernating animals emerge from torpor without neurological deficits or with enhanced cognitive function (37, 38). Frerichs et al. (16) showed that although cerebral blood flow decreases 80 to 90% during torpor, neurons are not adversely affected. The present study extends these observations to include animals in late arousal using early indicators of oxidative stress. In no case, was evidence of histopathology or oxidative modification of biomolecules observed in brain.

**Conclusion**

Arctic ground squirrels show evidence of hypoxia but no neuronal pathology, oxidative modification or cellular stress following the period of high metabolic demand necessary for arousal thermogenesis. In contrast, hibernating animals show no evidence of hypoxia, cellular stress, or inflammatory response in brain consistent with suppressed metabolic demand and immune responsiveness that likely contribute to the highly protective nature of hibernation. Finally, euthermic AGS experience mild, chronic hypoxia with low hemoglobin-oxygen saturation and accumulation of HIF 1α and iNOS, which demonstrate the greatest degree of cellular stress in brain. The significance of mild, chronic stress in euthermic AGS remains to be determined.
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8. **Castellani RJ, Harris PL, Sayre LM, Fujii J, Taniguchi N, Vitek MP, Founds H, Atwood CS, Perry G, and Smith MA.** Active glycation in neurofibrillary pathology of


Figure legends:

Figure 1: Arterial blood gases and other physical parameters in rats, euthermic and hibernating AGS. Hibernating AGS have normal blood gas levels ($P_aO_2$: 81-132mmHg; $P_aCO_2$: 25-30mmHg; pH: 7.49-7.60) while euthermic AGS are slightly hypoxic ($P_aO_2$: 41-73mmHg; $P_aCO_2$: 30-84mmHg; pH: 7.32-7.46) compared to rats ($P_aO_2$: 77-91 mmHg; $P_aCO_2$: 42-48mmHg; pH: 7.45-7.48) and hibernating AGS. Horizontal bars show significant differences ($p<0.05$) between groups. Animal number: $n= 5$ for rats, 8 for euthermic AGS and 6 for hibernating AGS. Heart rate (HR) for hibernating AGS is 6.8 bpm.

Figure 2: Representative sample of arterial blood gas values and physiological parameters during arousal from hibernation shows that $P_aO_2$ reaches a minimum at the time of maximal oxygen consumption. Respiratory frequency (Resp. freq.), respiratory quotient (RQ), oxygen consumption ($O_2$ cons.), electromyographic activity (EMG act), mouth temperature ($T_{mouth}$), abdominal temperature ($T_{abd}$), rectal temperature ($T_{rec}$), ambient temperature ($T_a$).

Figure 3: Mean blood gas values, and other parameters during arousal at maximum hypoxemia (mid arousal) and during late arousal in AGS compared with euthermic and hibernation. Results demonstrate hypoxia and metabolic challenge (high O$_2$ demand) during mid-arousal. Data shown are from euthermic AGS ($n=8$) and hibernating AGS ($n=7$) during torpor, and arousal, at the time when $P_aO_2$ was minimal, and after $T_b$ reached 34 ºC.

Figure 4: Hemoglobin-oxygen saturation (sO$_2$) in AGS ($n=9$) and rats ($n=8$) under both inhalation conditions of 100% O$_2$ with isoflurane (1%), i.e., light anesthetic condition) and 21%
O₂ in room air. sO₂ was measured with a rectal probe using a pulse oximeter. sO₂ was monitored in another group of AGS during arousal from hibernation and the minimum sO₂ recorded during arousal is shown for comparison with euthermic levels. Horizontal line shows significant difference between two groups.

**Figure 5**: HIF1α is increased in euthermic and late arousal AGS forebrain, consistent with low PₐO₂ levels in these groups of animals. (A) HIF1α immunoprecipitated (IP) from forebrain homogenates of different groups were immunoblotted (IB) by the same HIF1α antibody. (B) Quantification of band density demonstrates that level of HIF1α is significantly higher in late arousal and euthermic animals compared with hibernating animals while no significant difference is noted between late arousal and euthermic groups. Further, HIF1α levels in rat are similar to that of hibernating AGS and significantly different from euthermic AGS. Results are shown as mean ± SEM. (*p<0.02, **p<0.005, ***p<0.05).

**Figure 6**: iNOS expression is induced in euthermic forebrain. (A) Representative results of immunoblots of forebrain homogenates probed with antibody against iNOS are shown. The same membrane was stripped and re-probed with antibody against actin as a loading control. (B) Quantification of iNOS, which is normalized to the levels of actin, shows a significant increase of iNOS in euthermic AGS compared with hibernating and late arousal AGS, while no significant difference between hibernating and late arousal AGS is noted. Results are shown as mean ± SEM. (*p<0.001, n=5-7)

**Figure 7**: No neuronal pathology or evidence of intraneuronal oxidative stress was observed during euthermy, hibernation or late arousal. Representative photographs of cortical neurons, pyramidal cells, from a total of 5 animals per group are shown in A, B, C and D for CML immunoreactivity, and in E, F, G and H. for HNE immunoreactivity. Scale bar is 20 μm. A, E.
is for euthermic; B, F is for hibernating; C, G is for late arousal AGS; D, H is for positive control. Positive controls show CML immunoreactivity within neurons (arrow) and HNE immunoreactivity within neuronal membranes (arrow) and processes (star).
Fig. 1
Fig. 2.
Fig. 3.

Euthermic AGS
Hibernating AGS
AGS during mid-arousal at maximum hypoxemia
AGS after late-arousal when Tb reached 34 °C.
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.

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**CML**

**HNE**