Absence of cellular stress in brain after hypoxia stress induced by arousal from hibernation in Arctic ground squirrels

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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 warm up 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 euthermia, hibernation, and arousal is less well characterized.

Arterial oxygen tension (PaO₂) 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, PaO₂ was not measured to address the relationship between blood and tissue oxygenation during euthermia, hibernation, and arousal. To characterize physiological challenges associated with arousal thermogenesis, we evaluated blood oxygenation during euthermia, hibernation, and arousal along with heart rate, respiratory rate, body temperature, and oxygen consumption as signs of metabolic demand.

In addition, brain levels of hypoxia-inducible factor (HIF)-1α 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 euthermia, hibernation, or arousal. Thus, in the present study, we 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 Arctic ground squirrels (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 (PaO₂) and hemoglobin oxygen saturation (SO₂) are 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. With these results taken together, heterothermic mammals appear to maintain a low level of chronic hypoxia during euthermia 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 Com-

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mittee. AGS (S. parryii) 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, ~40 miles south of the Toolik Field Station of the University of Alaska Fairbanks (68°38'N, 149°38'W; elevation 809 m) and were transported to Fairbanks, AL (permit obtained from Alaska Department of Fish and Game). AGS 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<sub>a</sub>) of 2°C and a 4:20-h light-dark cycle. Male Sprague-Dawley rats (6–7 mo of age at time of experiment) were purchased from Simonsen Laboratories (Gilroy, CA) and were transported by air to the University of Alaska Fairbanks. Rats were housed in groups of two to four at 20–21°C, with a 12:12-h light-dark cycle, and were 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 maintained in the same environmental chamber to avoid confounding effects of temperature. A T<sub>a</sub> of 2°C is mild for this Arctic species, whose 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 (September through April) that did not hibernate (most probably due to disturbance, because “nonhibernators” will hibernate on other years). Summer euthermic AGS consisted of AGS moved in mid-May to a Ta of 16–17°C with a 24:0-h light-dark cycle set to mimic natural lighting conditions. Animals were housed under these conditions for 2 wk before SO<sub>2</sub> was measured. Hibernation is defined as prolonged torpor. Hibernating AGS consisted of animals in prolonged torpor (3–7 days) with respiratory rates <6 respirations/min and body temperature (T<sub>b</sub>) near T<sub>a</sub> [where abdominal T<sub>b</sub> was monitored via radiotelemetry and inserted thermocouples into the rectum and cheek pouch. Abdominal temperature (T<sub>a</sub>) 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). Rectal (T<sub>rec</sub>) and mouth (T<sub>mouth</sub>) temperatures were recorded with copper-constantan thermocouples connected to thermocouple amplifiers (AD595; Analog Devices, 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). Respiratory rates were counted for 60 s in euthermic animals and for 10 s in hibernating animals by visually inspecting the animal before blood sampling. In this group, lactate was measured with an I-Stat blood gas analyzer and G4+ cartridges. Neither oxygen consumption nor glucose was measured. T<sub>b</sub> during arousal was between 1 and 3°C except in one animal whose T<sub>b</sub> was 21°C. Data obtained from groups A and B were 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<sub>2</sub>). Therefore, SO<sub>2</sub> was measured in winter and summer euthermic AGS and rats by using pulse oximetry (Vet/Ox TM 4402L, Sensor Devices, Waukesha, WI). A rectal probe was inserted under isoflurane anesthesia. Animals were lightly anesthetized with isoflurane mixed with 100% O<sub>2</sub> (induced at 5% and maintained at 1% isoflurane) and delivered at a rate of 1.5 l/min. Measurements for SO<sub>2</sub> and heart rate were taken while animals were breathing 100% O<sub>2</sub> and maintained on 1% isoflurane. Anesthesia and oxygen were then discontinued, and animals were allowed to breathe room air. Heart rate and SO<sub>2</sub> 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. SO<sub>2</sub> and heart rate were recorded...
within the operating range of the instrument (>6°C) and until the animal began to move. Trec was monitored with a thermistor.

Collection of tissues for immunoblotting, histopathology, and immunohistochemistry. In separate, nonoperated animals, tissue was sampled from four groups: rats, euthermic AGS, hibernating AGS, and late-arousal AGS (late-arousal AGS had reached a core Tb of at least 34°C; see Animals for more details). Before initial sampling, animals were lightly anesthetized with halothane (5% halothane mixed with O2 delivered at 1.5 l/min). Trec was measured with a thermistor. After 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 <10 min. The remaining cerebral hemisphere was fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid) and postfixed overnight. Forebrain was trimmed, paraffin embedded, and cut in 7-μm 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 vols of lysis buffer (50 mM Tris·HCl, pH 7.6, 0.02% sodium azide, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 150 mM NaCl, 1 mM phenylmethylsulfonxy fluoride, 1 μg/ml aprotinin, 1 μg/ml antipain, and 1 mM sodium orthovanadate). The protein concentration was determined by BCA assay (Pierce). Proteins were separated by SDS-PAGE (40 μg/lane) and electroblotted onto Immobilon-P membrane (Millipore, Bedford, MA) by standard procedures as previously described (64). Transferred blots were incubated sequentially with blocking agent [10% nonfat milk in Tris-buffered saline (TBS)-Tween], anti-iNOS antibody (polyclonal, made in rabbit; Santa Cruz Biotechnology, Santa Cruz, CA), and affinity-purified goat anti-rabbit immunoglobulin-peroxidase conjugate preabsorbed to eliminate human cross-reactivity. Blots were developed using the ECL technique (Santa Cruz Biotechnology) according to the manufacturer’s instructions. 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.

Immunocytochemistry. 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 vols of lysis buffer (50 mM Tris·HCl, pH 7.6, 0.02% sodium azide, 1% NP-40, 150 mM NaCl, 1 mM phenylmethylsulfony 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, Indianapolis, IN) at 4°C for 2 h, followed by centrifugation at 10,000 rpm for 10 min at 4°C. HIF-1α antibody (10 μl, 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 h with end-over-end rotation, followed by the addition of protein A-agarose and incubation overnight. After centrifugation at 10,000 rpm for 10 min at 4°C, the supernatant was carefully aspirated and discarded. The pellet was washed four times with lysis buffer, and the sample was boiled for 10 min before SDS-PAGE was performed. The entire precipitate was loaded in one lane, and proteins were separated by SDS-PAGE followed by transfer onto Immobilon-P membrane (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 were processed immunohistochemically using carboxymethyl 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, after 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 TBS (50 mM Tris·HCl, 150 mM NaCl, pH 7.6) to reduce nonspecific binding. After being rinsed 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) antisera, followed by species-specific peroxidase anti-peroxidase complex (Dianova, Monoclonals and ICN Capel). 3-3′-Diaminobenzidine tetrahydrochloride was used as a chromagen. Hippocampal sections from Alzheimer’s disease cases, studies of which 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), Harris hematoxylin stain, and Papanicolaou EA-50 (Anpath) were obtained from StatLab (Lewisville, TX).

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

RESULTS

Blood gas analyses show that PaO2, from hibernating AGS are significantly higher than from normoxic rats. Arterial CO2 tension (Paco2) tended to be lower and pH was significantly higher, consistent with suppressed CO2 production (Fig. 1). In contrast, euthermic AGS appear mildly hypoxic, as indicated by low PaO2, and pH and high Paco2, all of which are significantly different from values in hibernating AGS and/or rats (with the exception of Paco2) (Fig. 1). Paco2 values in euthermic AGS were inversely and significantly correlated with PaO2 values (P < 0.05; R2 = 0.55).

PaO2 was decreased significantly during arousal. Figure 2 shows a sample from one AGS illustrating a drop in PaO2 at the time of peak oxygen consumption. Respiratory frequency increased at the 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, Paco2 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, Paco2 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 with euthermia at the end of arousal and increased briefly just after maximal oxygen consumption.

Figure 3 shows means from seven AGS monitored throughout arousal as well as the group of euthermic AGS from Fig. 1 for comparison. PaO2 consistently dropped to a minimum during arousal. At this time, Paco2 did not change compared
Fig. 1. Arterial blood gases and other physiological parameters measured in rats and euthermic and hibernating Arctic ground squirrels (AGS). Hibernating AGS have normal blood gas levels \((P_{O_2}, 81–132 \text{ mmHg}; P_{CO_2}, 25–30 \text{ mmHg}; \text{pH} 7.49–7.60)\), whereas euthermic AGS are slightly hypoxic \((P_{O_2}, 41–73 \text{ mmHg}; P_{CO_2}, 30–84 \text{ mmHg}; \text{pH} 7.32–7.46)\) compared with rats \((P_{O_2}, 77–91 \text{ mmHg}; P_{CO_2}, 42–48 \text{ mmHg}; \text{pH} 7.45–7.48)\) 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 beats/min (bpm). \(P_{O_2}\), arterial \(P_{O_2}\); \(P_{CO_2}\), arterial \(P_{CO_2}\); \(T_b\), body temperature; RR, respiratory rate.

Heart rate, recorded at the same time as \(S_{O_2}\), 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/min \((n = 9, \text{body weight} 731 ± 40 \text{ g})\). The mean heart rate in rats breathing 100% \(O_2\) and room air was 347 ± 3 and 344 ± 3 beats/min \((n = 8, \text{body weight} 366 ± 8 \text{ g})\). There was no significant difference in heart rate between winter and summer euthermic AGS under any condition \((P > 0.05)\).

During arousal from hibernation, the mean minimum \(S_{O_2}\) in AGS was 57 ± 10% \((n = 5, \text{body weight} 803 ± 45 \text{ g})\) (Fig. 4). \(T_{rec}\) was 10 ± 2°C and heart rate was 42 ± 6 beats/min at the time minimum \(S_{O_2}\) was recorded. There was no significant difference in body weight among winter, summer, and midarousal AGS in which \(S_{O_2}\) was measured \((P > 0.05)\).

To begin to relate arterial blood gas levels to tissue oxygenation, we measured protein levels of HIF-1α in forebrain as an indicator of tissue oxygenation. Consistent with low \(P_{O_2}\) in euthermic and late-arousal AGS, HIF-1α was significantly higher in these two groups of animals compared with hibernating AGS or normoxic rats (Fig. 5).

To explore the intracellular consequence of arousal reperfusion/thermogenesis in brain, we measured the levels of iNOS (Fig. 6). Indeed, iNOS expression was hardly detectable in hibernating animals, whereas it was dramatically induced (2.5-fold increase) in euthermic animals. However, the expression of iNOS was 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). Alzheimer’s disease tissue, run in parallel as a positive control, showed dark immunoreactive product with both CML and HNE antibodies. Furthermore, there was no
Overt neuronal pathology observed in H&E-stained sections from late-arousal animals (not shown).

**DISCUSSION**

Hibernation provides significant neuroprotection (14, 63) that is beginning to be appreciated for multifaceted mechanisms that may be linked to other models of ischemia and hypoxia tolerance (12, 55). 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 after arousal, without evidence of cellular stress.

**Hypoxia during euthermy.** PaO₂ values of 80 mmHg have been reported in euthermic animals of other heterothermic species (5, 15, 29, 40), although such low PaO₂ levels have not been observed consistently (16, 56). Low PaO₂, high PaCO₂, and low pH have 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. Although 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 values in summer and winter AGS (83–85%) further support the interpretation that this species sustains mild, chronic hypoxia. Rats, like humans and most other mammals, maintain SO₂ values of 95% or greater, and an SO₂ value <95% is an indication for supportive therapy (18, 28, 45). The SO₂ results reported in the present study 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 PaO2 of 50 mmHg corresponds to an SO2 of ~90% (34, 40). Alternatively, if the curve is left shifted in AGS, other factors such as high PaCO2 and low pH observed in AGS may contribute to low SO2 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% O2 (equivalent to a PaO2 of 60 mmHg in rat) is sufficient to increase brain levels of HIF-1α. We measured for the first time HIF-1α levels in the brain of heterothermic mammals as an indicator of tissue oxygen 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

Fig. 3. Mean blood gas values and other parameters measured during arousal at maximum hypoxemia (midarousal) and during late arousal in AGS compared with euthermic and hibernation. Results demonstrate hypoxia and metabolic challenge (high O2 demand) during midarousal. Data are from euthermic AGS (n = 8) and hibernating AGS (n = 7) during torpor and arousal at the time when PaO2 was minimal and after Tb reached 34°C. Horizontal bars show significant differences (P < 0.05) between groups. VO2, O2 consumption.

Fig. 4. Hemoglobin O2 saturation (SO2) measured in AGS (n = 9) and rats (n = 8) under both inhalation conditions of 100% O2 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 bars show significant differences (P < 0.05) between groups.

Fig. 5. Hypoxia-inducible factor (HIF)-1α is increased in euthermic and late-arousal AGS forebrain, consistent with low PaO2 levels in these groups of animals. A: HIF-1α immunoprecipitated (IP) from forebrain homogenates of different groups were immunoblotted (IB) by the same HIF-1α antibody. B: quantification of band density demonstrates that the level of HIF-1α is significantly higher in late-arousal and euthermic animals compared with hibernating animals, whereas no significant difference is noted between late-arousal and hibernating groups. Furthermore, HIF-1α levels in rats are similar to those in hibernating AGS and significantly different (*P < 0.02, **P < 0.005, ***P < 0.05) from euthermic AGS. Results are shown as means ± SE.
that a recent report showed that elevated HIF-1α levels were correlated with activation of JNK and ERK (65).

In addition to evidence of tissue hypoxia, elevated iNOS, a marker of cellular stress (23, 24, 44) 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). On the basis of the present results, a difference in SO2 cannot explain the greater p38 activation in summer euthermic AGS brain, because SO2 was not different between winter and summer euthermic AGS.

**Normoxia during hibernation.** PaO2 and PaCO2 in hibernating AGS are similar to values in other reports (15, 40) and consistent with pronounced metabolic suppression and decreased CO2 production during hibernation (4). Elevated blood pH is consistent with one reported result (56) but differs from most other studies in which pH of hibernating and euthermic ground squirrels do not differ (15, 16, 27, 29, 40). Persistently high blood pH in hibernating AGS, furthermore, contrasts with acid-base regulation in homeothermic mammals. A similar decrease in PaCO2 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 af-

Fig. 6. Inducible nitric oxide synthase (iNOS) expression is induced in euthermic forebrain. A: representative results of immunoblots of forebrain homogenates probed with antibody against iNOS. The same membrane was stripped and reprobed with antibody against actin as a loading control. H1 and H2, hibernating forebrain; E1 and E2, euthermic forebrain; A1 and A2, late-arousal forebrain. B: quantification of iNOS, which is normalized to the levels of actin, shows a significant (*P < 0.001; #P < 0.005) increase of iNOS in euthermic AGS compared with hibernating and late-arousal AGS, whereas no significant difference between hibernating and late arousal AGS is noted. Results are shown as means ± SE; n = 5–7.

Fig. 7. No neuronal pathology or evidence of intraneuronal oxidative stress was observed during euthermy, hibernation, or late arousal. Representative photographs of cortical, pyramidal neurons from a total of 5 animals per group, are shown in A–D for carboxymethyl lysine (CML) immunoreactivity and in E–H for 4-hydroxy-2-nonenal (HNE) immunoreactivity. Scale bar, 20 μm. Positive controls show CML immunoreactivity within neurons (arrow) and HNE immunoreactivity within neuronal membranes (arrow) and processes (star).
finity for H+ increases at colder temperatures, causing pH to change ~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 a higher value. For example, whereas 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 SO2 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 PaO2 falling to 9 mmHg and lasting 4 h and SO2 reaching a minimum of 57%. Although unloading of oxygen should be enhanced during warming and transition to euthermia (34), oxygen 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-1α in brain during late arousal are consistent with brain tissue hypoxia. Rat brain cortex rapidly accumulates HIF-1α during the onset of exposure to 10% O2 (10), suggesting that the time course of tissue sampling is sufficient to observe an increase in HIF-1α 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 one-half (from 10 to 5 mM) 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 (11, 58). 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% O2 increases arterial blood lactate concentrations threefold or more (Ma YL, Cozad KD, Rivera PM, Zhao, HW, and 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 are generated during reperfusion, hypoxia, and reoxygenation and cause oxidative damage to important cellular components that contributes to cell death (2, 3, 22, 53, 57). We therefore examined brain tissues from euthermic, hibernating, and late-arousal AGS for cellular damage and oxidative modification. Prior studies showed that lipid peroxidation and protein oxidation occur 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, 33, 58).

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 80–90% during torpor, neurons are not adversely affected. The present study extends these observations to include animals in late arousal by using early indicators of oxidative stress. In no case was evidence of histopathology or oxidative modification of biomolecules observed in brain.

In 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 Arctic ground squirrels 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 Arctic ground squirrels remains to be determined.
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