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Metabolism of Brain Cortex and Cardiac Muscle Mitochondria in Hibernating 13-Lined Ground Squirrels *Ictidomys tridecemlineatus*

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ABSTRACT

During bouts of torpor, mitochondrial metabolism is known to be suppressed in the liver and skeletal muscle of hibernating mammals. This suppression is rapidly reversed during interbout euthermic (IBE) phases, when whole-animal metabolic rate and body temperature (T_b) return spontaneously to euthermic levels. Such mitochondrial suppression may contribute significantly to energy savings, but the capacity of other tissues to suppress mitochondrial metabolism remains unclear. In this study we compared the metabolism of mitochondria from brain cortex and left ventricular cardiac muscle between animals sampled while torpid (stable T_b near 5°C) and in IBE (stable T_b near 37°C). Instead of isolating mitochondria using the traditional methods of homogenization and centrifugation, we permeabilized tissue slices with saponin, allowing energetic substrates and inhibitors to access mitochondria. No significant differences in state 3 or state 4 respiration were observed between torpor and IBE in either tissue. In general, succinate produced the highest oxidation rates followed by pyruvate and then glutamate, palmitoyl carnitine, and β -hydroxybutyrate. These findings suggest that there is no suppression of mitochondrial metabolism or change in substrate preference in these two tissues despite the large changes in whole-animal metabolism seen between torpor and IBE.

Introduction

Many small mammals have adopted hibernation as a strategy to cope with the high thermogenic demands and low food availability of cold winter months. Hibernation consists of repeated bouts of torpor, each of which is spontaneously interrupted by periods of euthermia. During entrance into a torpor

bout in the 13-lined ground squirrel *Ictidomys tridecemlineatus*, whole-animal metabolic rate decreases rapidly to less than 10% of euthermic levels, and body temperature (T_b) decreases over several hours from 37° to approximately 5°C. The torpid stage, characterized by low and stable levels of T_b and metabolic rate, is maintained for 6–12 d under laboratory conditions and ends with the arousal phase, during which metabolic rate and T_b increase over several hours. Arousals lead to periods of interbout euthermia (IBE), where T_b and metabolic rate are sustained at euthermic levels for approximately 8 h in this species (Brown et al. 2012).

Reductions in mitochondrial metabolism are evident in many animals that are capable of surviving environmental stresses that can limit the capacity to produce adenosine triphosphate (ATP) aerobically (reviewed in Staples and Buck 2009). As the major site of cellular oxygen consumption and heat production, mitochondria have been the focus of many attempts to understand the nature of the reversible metabolic suppression seen in hibernators. Between IBE and torpor, the respiration rates of isolated liver mitochondria (measured at 37°C with succinate as substrate) decline by up to 70% (Armstrong and Staples 2010; Chung et al. 2011). Because the liver accounts for 12%–17% of whole-body metabolism in rodents (Martin and Fuhrman 1955), such suppression of mitochondrial metabolism could result in significant energy savings over the course of a hibernation season. However, there is little evidence for such suppression in other tissues. Earlier studies found no significant difference in respiration rates of isolated skeletal muscle mitochondria when torpid ground squirrels were compared with summer active animals (Barger et al. 2003; Muleme et al. 2006). Such comparisons, however, may be confounded by seasonal differences (summer vs. winter) rather than by changes associated with torpor per se (Martin and Epperson 2008). One of our recent studies avoided potential seasonal effects by comparing skeletal muscle mitochondrial metabolism between winter animals sampled either in torpor or IBE and found 33% suppression in torpor (Brown et al. 2012). The suppression in muscle is modest compared with liver, but because skeletal muscle is estimated to contribute 25% to basal metabolic rate (in rats of similar mass to ground squirrels; Martin and Fuhrman 1955), significant energy savings could be realized over a hibernation season. Data from other tissues are limited (reviewed in Staples and Brown 2008), so the goal of this study was to assess the degree, if any, of mitochondrial metabolic suppression during ground squirrel hibernation in heart and brain cortex.

One might predict metabolic suppression in heart, as a muscular tissue, similar to that observed in skeletal muscle mitochondria during the torpid stage (Brown et al. 2012). Skeletal

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muscle generally remains inactive throughout the torpid stage, but it shivers briefly during arousal. Cardiac muscle, on the other hand, must continue to work during torpor, if at lower levels. During entrance, the transition from IBE to torpor, heart rate decreases from as high as 450 to as low as 5 beats min^{-1} in *I. tridecemlineatus* (Hampton et al. 2010), and cardiac output falls to a similar degree (Popovic 1964). Such decreases may be considered greater than expected solely because passive thermal effects of a falling T_b —for heart rate $Q_{10} = 4.5$ —so one might hypothesize that active metabolic suppression occurs in this tissue during torpor. Such metabolic suppression, if occurring, would likely be reflected in mitochondrial metabolism. Therefore, we compared heart mitochondrial metabolism between torpor and IBE.

Ground squirrel hypothalamus and brain cortex undergo changes in glucose uptake during the transitions between torpor and IBE (Kilduff et al. 1990), but brain wave activity declines during entrance, and below a T_b of 10°C, electroencephalogram traces are virtually silent (Walker et al. 1977). A reduction in brain cortex metabolism in torpor is suggested by several previous findings. There is a significant decrease in glycogen phosphorylase activity in brains of hibernating hamsters (Lust et al. 1989). Glucose uptake by torpid ground squirrel brain is reduced to 1%–2% of active control values (Frerichs et al. 1995). Ground squirrel brain also appears to be inherently resistant to ischemia, and reducing oxygen demand by suppressing mitochondrial oxidative phosphorylation would facilitate such resistance (Dave et al. 2012). On the other hand, high-energy phosphates in the brains of ground squirrels appear to be higher in torpor than IBE (Henry et al. 2007), suggesting that ATP consumption is downregulated to a greater degree than ATP production. We compared mitochondrial metabolism in the brain cortex between torpor and IBE to provide further insight into the potential energy savings occurring in this tissue and perhaps to advance understanding of the mechanisms of ischemia resistance in hibernators.

Data from whole-animal metabolism and tissue enzymes suggest a shift in oxidative substrate preference between torpor and IBE. Respiratory quotient values near 0.7 suggest that steady state torpor is fueled almost exclusively by lipid oxidation in arctic ground squirrels but shifts toward carbohydrate oxidation during IBE (Karpovich et al. 2009). Concentrations of ketone bodies in blood increase almost 10-fold between the fed summer condition and winter IBE in Belding's ground squirrel, with a further increase in the winter torpid state (Krilowicz 1985). Enzymes important to carbohydrate oxidation—such as phosphofructokinase, pyruvate kinase, and pyruvate dehydrogenase—are covalently modified during torpor in hibernating ground squirrel heart, drastically reducing maximal activities (Brooks and Storey 1992; Storey 1998). The activity of acetyl-CoA carboxylase decreases significantly in hibernating and IBE Richardson's ground squirrel hearts (Belke et al. 1998). The product of acetyl-CoA carboxylase, malonyl CoA, inhibits β -oxidation. Taken together these observations suggest that during torpor there is a shift away from carbohydrate oxidation toward oxidation of lipid metabolites. We evaluated potential

changes in mitochondrial substrate preference by providing several different oxidative substrates to mitochondria from brain cortex and heart left ventricle.

Material and Methods

Animals

All experiments were approved by the Animal Use Subcommittee of the University of Western Ontario (protocol 2008-055). Thirteen-lined ground squirrels *Ictidomys tridecemlineatus* were livetrapped in Carman, Manitoba (49°29'57"N, 98°0'3"W). Some animals were born in captivity to wild-caught dams that were impregnated in the wild. Animal husbandry followed methods recently described (Brown et al. 2012). Squirrels were housed at $22^\circ \pm 3^\circ\text{C}$ and a photoperiod matching that of Carman, Manitoba, for the time of year (adjusted weekly) until October. In early October, animals were moved to environment chambers where temperature was reduced 1°C d^{-1} until it reached $4^\circ \pm 2^\circ\text{C}$, at which point photoperiod was reduced to 2L:22D, with lights on at 0815 hours. Food and water remained available ad lib. until torpor was observed (typically within 1 wk), at which time food was withdrawn. Hibernation state was determined by T_b , which was monitored continuously using implanted radio telemeters as described previously (Muleme et al. 2006). IBE animals had spontaneously aroused and maintained a stable T_b near 37°C for 1–4 h before they were euthanized by anesthetic overdose (Euthanyl, 270 mg mL^{-1} , 0.2 mL 100 g^{-1}). Euthanyl has previously been shown to have no effects on mitochondrial metabolism (Takaki et al. 1997). As anesthetic injection would cause arousal, torpid squirrels were euthanized by cervical dislocation.

Tissue Permeabilization

Previous studies used isolated and purified mitochondria from liver (Armstrong and Staples 2010; Chung et al. 2011) and skeletal muscle (Brown et al. 2012) to assess mitochondrial metabolism in hibernators. This traditional technique relies on tissue homogenization and repeated centrifugation steps, which can result in very low mitochondrial yields and precludes its use with small tissue samples. A novel aspect of this study was the use of saponin-permeabilized tissue slices, allowing us to assess mitochondrial function using very small tissue samples. Moreover, the permeabilization technique reduced the time required to process tissue, presumably minimizing disruption of mitochondrial metabolism.

Immediately following euthanasia, the brain and heart were excised, and each was placed in 15 mL of ice-cold BIOPS solution (20 mM taurine, 15 mM phosphocreatine, 20 mM imidazole, 0.5 mM DTT, 10 mM Ca^{2+} EGTA, 5.77 mM ATP, 6.56 mM MgCl_2 , 50 mM K^+ -MES, pH 7.0; Kuznetsov et al. 2008). Within 10 min of excision, the brain cortex was then dissected into several longitudinal pieces (1 mm \times 1 mm \times 2 mm, approximately 25 mg each) using single-sided razor blades. Each piece was placed into 3 mL of ice-cold BIOPS in a 12-well tissue culture plate, covered, and gently agitated (30 rpm)

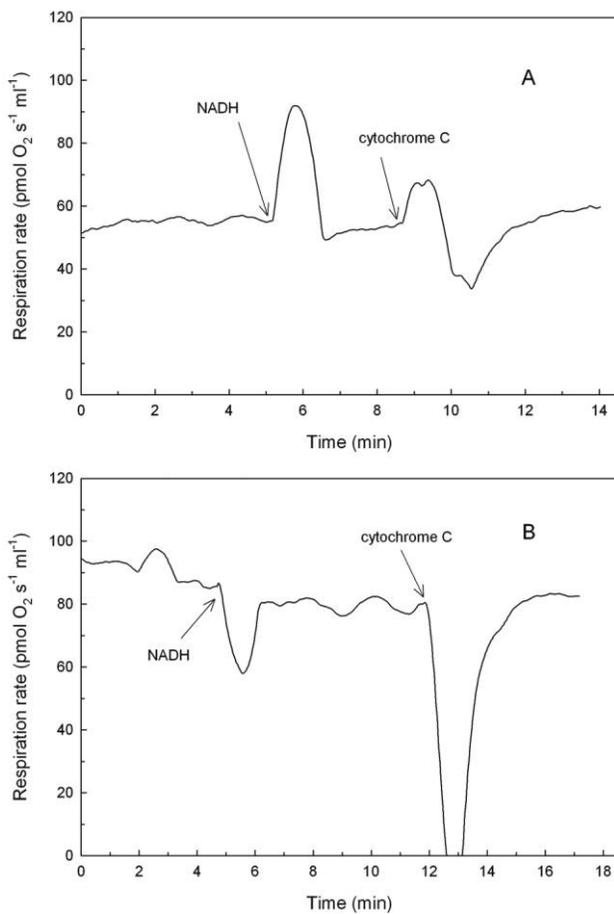


Figure 1. Representative examples of respiration of saponin-permeabilized brain cortex (A) and heart left ventricle (B). Respiration rate was measured without substrate and following the addition of NADH (0.2 mM) and cytochrome c (10 μ M) to assess the effect of the permeabilization protocol on the integrity of the inner and outer mitochondrial membranes, respectively.

on ice for 5 min using a metabolic shaker, following published techniques (Benani et al. 2009).

The cardiac tissue was prepared using a modification of a previously described technique (Kuznetsov et al. 2008). In a petri dish of ice-cold BIOPS, the left ventricle of the heart was cut lengthwise into small (approximately 5 mg) samples using a scalpel. All connective tissue was removed, and the fiber bundles were mechanically teased apart using fine sharp forceps under a dissection microscope with a chilled stage. Once the bundles were separated, each tissue sample was placed in 3 mL of ice-cold BIOPS in a 12-well tissue culture plate, covered, and gently agitated on ice for 5 min.

After incubation in BIOPS for 5 min on ice, 30 μ L of freshly prepared saponin solution (5 mg mL⁻¹ in BIOPS) was added to each well containing a tissue sample. Both brain and heart tissue slices from the same animal were then incubated on ice with gentle agitation for 30 min. Subsequently, the tissues were rinsed in 5 mL of mitochondrial respiration medium (0.5 mM

EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, 1 g L⁻¹ fatty acid-free bovine serum albumin, pH 7.1; Kuznetsov et al. 2008) three times for 5 min each. The wet mass of each sample was then determined, following blotting on filter paper, using a microbalance (MX5, Mettler Toledo, Columbus, OH).

Mitochondrial Respiration

Following permeabilization, tissue slices were transferred to chambers of an Oxygraph-2K high-resolution respirometer (Oroboros Instruments) containing 2 mL of mitochondrial respiration medium under constant stirring (750 rpm) at 37°C, the temperature where maximal mitochondrial suppression is evident in liver (Muleme et al. 2006) and skeletal muscle (Brown et al. 2012) from torpid ground squirrels. The medium was rendered hyperoxic (approximately 350 nmol mL⁻¹) to ensure adequate oxygen supply over the relatively large diffusion distances of tissue slices compared with isolated mitochondria. Hyperoxygenation was achieved by introducing gaseous oxygen above stirred medium within respirometer chambers. Oxygen was replenished if the medium concentration fell below approximately 200 nmol mL⁻¹.

Before measuring respiration rates with typical mitochondrial substrates, we added NADH (0.2 mM) and cytochrome c (10 μ M) to tissue preparations to assess the quality of the inner and outer mitochondrial membranes, respectively. Basal

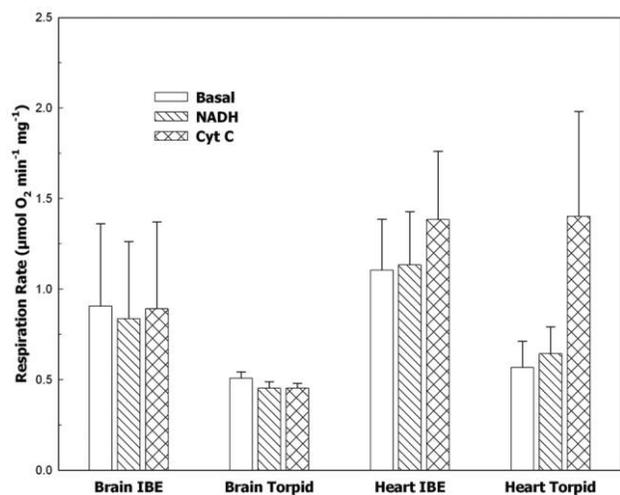


Figure 2. Respiration of saponin-permeabilized brain cortex and heart left ventricle tissue without substrate (basal), with NADH (0.2 mM), or with cytochrome c (cyt c; 10 μ M). Tissues were sampled from either torpid ground squirrels ($T_b \sim 5^\circ\text{C}$) or animals in interbout euthermia (IBE; $T_b \sim 37^\circ\text{C}$), and respiration was determined in vitro at 37°C. Values are mean \pm SEM for each treatment. Within each tissue and hibernation condition, the addition of NADH or cyt c had no significant effect on respiration rate, indicating that the integrity of both the inner and outer mitochondrial membranes was maintained throughout the permeabilization process. Sample sizes: brain IBE, $n = 4$; brain torpid, $n = 5$; heart IBE, $n = 4$; heart torpid, $n = 5$.

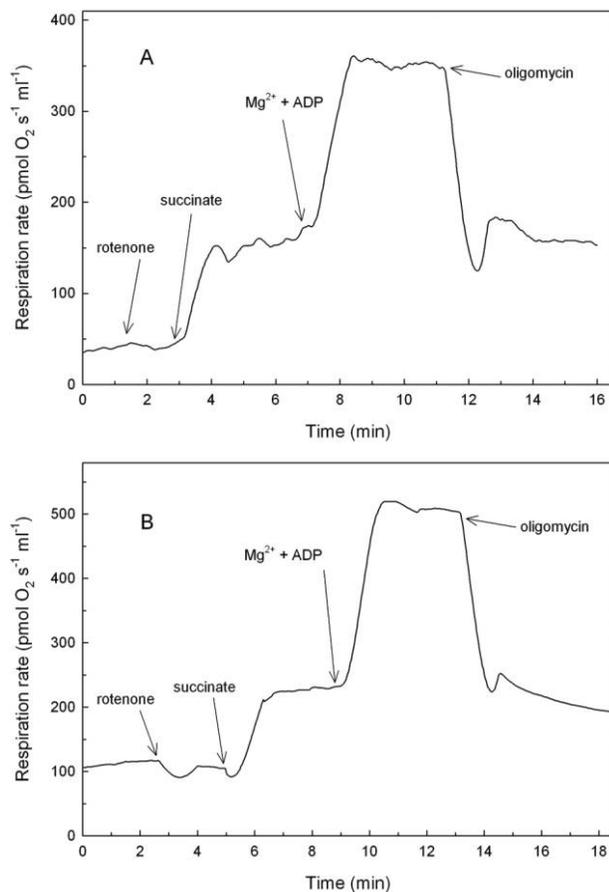


Figure 3. Representative examples of saponin-permeabilized brain cortex (A) and heart left ventricle (B) used to assess state 3 and state 4 mitochondrial respiration. After stable basal rates were established, rotenone ($0.5 \mu\text{M}$ in ethanol) and succinate (30 mM) were added. After steady state 2 respiration rates were established, ADP and Mg^{2+} (5 mM) were added to stimulate state 3 respiration. Oligomycin ($160 \mu\text{g mL}^{-1}$, in ethanol) was then added to estimate state 4 respiration.

(state 1) respiration was measured for each tissue slice in the absence of added substrates followed by the addition of substrates to determine state 2 respiration rates. State 3 respiration was stimulated in the tissue by addition of adenosine 5'-diphosphate (ADP) and Mg^{2+} (5 mM). State 4 respiration (non-phosphorylating) was estimated by addition of oligomycin ($160 \mu\text{g mL}^{-1}$ final concentration added in $20 \mu\text{L}$ ethanol). The substrates added were succinate (30 mM), pyruvate (10 mM), glutamate (30 mM), β -hydroxybutyrate (10 mM), and palmitoyl carnitine ($10 \mu\text{M}$). Malate (4 mM) was added with all substrates except succinate to ensure Krebs cycle intermediates were not limiting to mitochondrial respiration. Rotenone ($0.5 \mu\text{M}$ final concentration, added in $20 \mu\text{L}$ ethanol) was added along with succinate. Unless otherwise stated, all added chemicals were dissolved in mitochondrial respiration medium. Respiration rates were recorded and calculated as the first derivative of oxygen concentration in the respirometer chambers using Datalab 4 software (Oroboros Instruments).

Analysis

To determine the integrity of mitochondrial membranes within each tissue and hibernation state in the presence of NADH and cytochrome c by one-way ANOVA. State 3 and state 4 respiration rates were analyzed by two-way ANOVA with hibernation state (torpor or IBE) and substrates as factors. Student-Newman-Keuls post hoc tests were used to identify differences where the ANOVA P values were <0.05 .

Results

The addition of NADH or cytochrome c did not significantly alter basal respiration rates in either heart or brain (figs. 1, 2), indicating that the permeabilization protocol did not affect the integrity of the inner or outer mitochondrial membranes. Both tissues showed strong responses to the addition of ADP (with Mg^{2+}) and oligomycin (fig. 3), suggesting good mitochondrial respiratory control. The mass of brain tissue ranged from 17.2 to 40.0 mg , and the mass of heart tissue ranged from 3.0 to 6.0 mg . Despite these fairly large ranges, ANCOVA showed that tissue mass did not affect mass-specific state 3 respiration with succinate as a substrate (data not shown).

For the brain, neither state 3 nor state 4 respiration rates differed significantly between IBE and torpor, regardless of substrate (fig. 4). Similarly, for the heart ventricle, there was no significant effect of hibernation state on either state 3 or state 4 respiration rates regardless of substrate (fig. 5). There was no significant interaction between hibernation state and substrate in either tissue.

Oxidative substrate significantly affected respiration rates in each tissue. In brain cortex tissue, succinate was oxidized at a higher rate than palmitoyl carnitine, glutamate, and β -hydroxybutyrate in both state 3 ($P = 0.016$) and state 4 ($P < 0.001$; fig. 4) respiration, but rates with pyruvate did not differ significantly from any other substrate. In the heart, state 3 succinate oxidation was higher than pyruvate ($P = 0.02$) and palmitoyl carnitine, β -hydroxybutyrate, and glutamate ($P < 0.001$; fig. 5A). This trend was also observed in heart state 4 respiration, with succinate oxidized at a significantly higher rate ($P < 0.001$), but pyruvate was not preferentially oxidized relative to the other three substrates (fig. 5B).

Discussion

Saponin permeabilization of tissue from brain cortex and heart left ventricle allowed us to compare mitochondrial metabolism between torpid and IBE ground squirrels using very small amounts of tissue. Respiration rates reported in this study, measured in vitro at 37°C , compare favorably with those reported from rat brain cortex (Benani et al. 2009) and heart (Kuznetsov et al. 2004) at 30°C , indicating that this technique is valid for our purposes.

There is significant suppression of both liver (Muleme et al. 2006; Armstrong and Staples 2010; Chung et al. 2011) and skeletal muscle (Brown et al. 2012) in torpid 13-lined ground

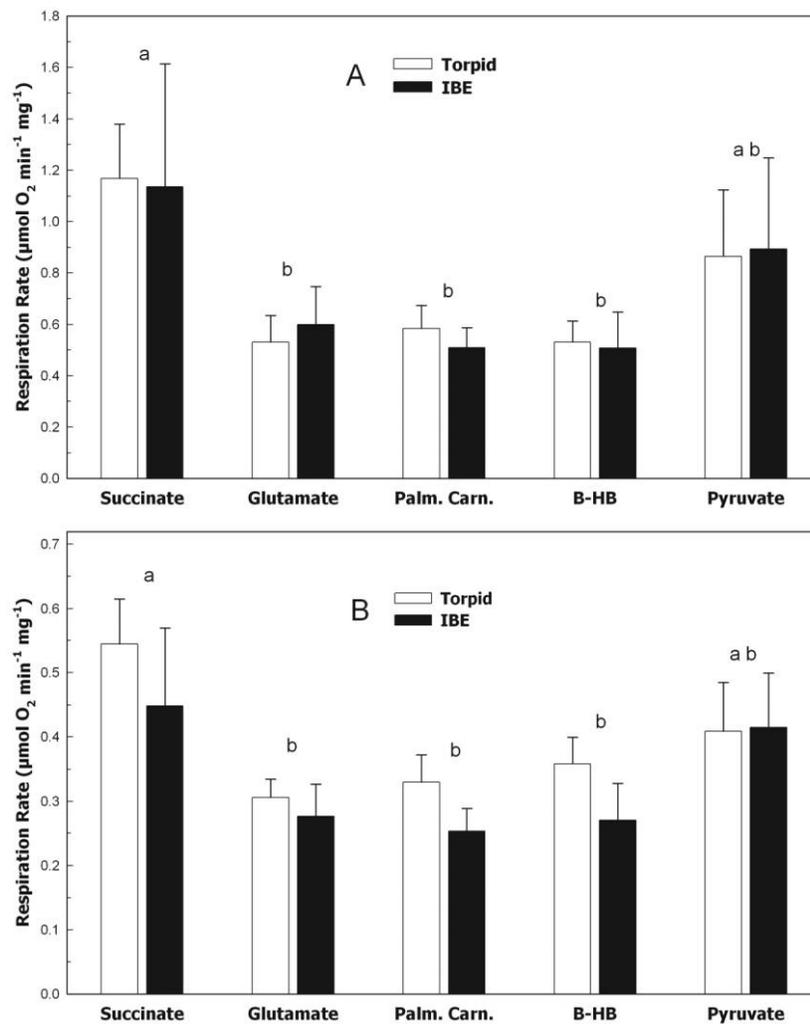


Figure 4. State 3 (A) and state 4 (B) respiration rates of saponin-permeabilized brain cortex tissue sampled from torpid ($n = 6$) or interbout euthermic (IBE; $n = 4$) ground squirrels oxidizing different substrates. Values are mean \pm SEM for each treatment. There is no effect of hibernation state on respiration regardless of oxidative substrate. Within each panel, substrate groups that share any letter label are not significantly different from each other, and there was no interaction between hibernation condition and substrate (two-way ANOVA). Palm. Carn. = palmitoyl carnitine; B-HB = β -hydroxybutyrate.

squirrels. The data in our study, however, do not support the hypothesis that there is active suppression of brain and heart mitochondrial metabolism during the transition between IBE and torpor. In our opinion this finding may result from differential regulation of tissue metabolism in hibernation and perhaps the heterogeneous nature of brain cortex and its metabolic response to hibernation.

We found no difference in brain cortex state 3 or state 4 respiration rates between torpor and IBE. Uptake of a glucose homologue has been used to examine ground squirrel brain metabolism in different stages of torpor bouts (Kilduff et al. 1990). Some cortex regions showed increases in relative glucose uptake, while other regions showed decreases or no change (Kilduff et al. 1990). If similar heterogeneous responses occur in cortex mitochondria, differences between hibernation states for the whole tissue may have been masked in our data. It

would be informative for future studies to separate cortical regions—particularly the cingulate region, which shows a marked change in relative glucose throughout a hibernation bout (Kilduff et al. 1990)—and analyze the responses of mitochondrial metabolism.

The freshwater turtle *Trachemys scripta* can survive extended periods of anoxia that would kill most other animals and is used as a model of cardiac muscle in energy-stressed states. A recent study using permeabilized cardiac muscle fibers found a significant suppression of mitochondrial state 3 and state 4 respiration in hearts from anoxic turtles compared with normoxic controls (Richards and Galli 2012). The data from this study, using very similar methods, found no change in these mitochondrial parameters between hearts from torpid ground squirrels and those from IBE ground squirrels. Although the heart rate of *Ictidomys tridecemlineatus* decreases considerably

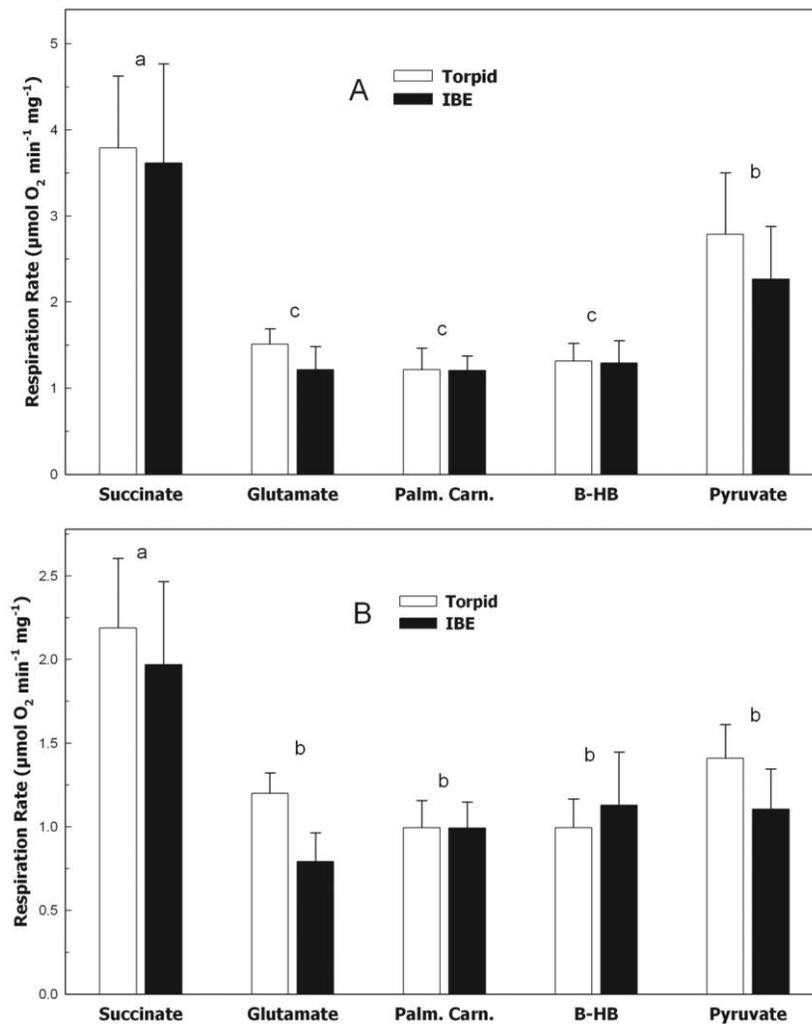


Figure 5. State 3 (A) and state 4 (B) respiration rates of saponin-permeabilized heart left ventricle tissue sampled from torpid ($n = 5$) or interbout euthermic (IBE; $n = 4$) ground squirrels oxidizing different substrates. Values are mean \pm SEM for each treatment. There is no effect of hibernation state on respiration regardless of oxidative substrate. Within each panel, substrate groups with different labels are significantly different from each other, and there was no interaction between hibernation condition and substrate (two-way ANOVA). Palm. Carn. = palmitoyl carnitine; B-HB = β -hydroxybutyrate.

in torpor (Hampton et al. 2010), isolated hearts from torpid ground squirrels can generate higher pressures than those of winter euthermic controls (Carpette and Senturia 1984). It is possible that mitochondrial oxidative capacities must be maintained in torpor to permit the high pressures required to pump blood that becomes more viscous at the low T_b experienced in torpor (Maclean 1981).

Previous data suggest changes in oxidative fuel use among hibernation states at the level of the whole animal (Krilowicz 1985; Buck and Barnes 2000) and tissue enzymes (Brooks and Storey 1992, 1998; Belke et al. 1998). Despite these findings we found no effect of torpor state on the rate of mitochondrial oxidation for pyruvate (derived from glycolysis), palmitoyl carnitine (a fatty acid derivative that can be transported into mitochondria), β -hydroxybutyrate (a ketone body produced especially under conditions of high rates of fatty acid catabolism),

succinate (a Krebs cycle intermediate oxidized directly by electron transport chain complex II), or glutamate (from amino acid metabolism) in either brain or heart tissue. These data differ somewhat from those reported for *I. tridecemlineatus* liver mitochondria, where succinate, pyruvate, and β -hydroxybutyrate oxidation rates appear to increase in euthermic animals (Muleme et al. 2006). These results suggest that oxidative substrate preference is regulated in a tissue-specific manner in hibernators.

The findings of this study suggest that unlike liver and skeletal muscle, no active suppression of cardiac muscle or brain cortex metabolism occurs during the transition from euthermia to torpor. The brain and heart are estimated to contribute only 5% (Hofman 1983) and <1% (Martin and Fuhrman 1955), respectively, to whole-body metabolic rate in rodents. Therefore, selective pressures favoring active suppression in these

tissues would be weak, resulting in only minimal energetic savings over the course of a hibernation season. These tissues may, however, serve as useful internal comparisons with tissues such as liver that do undergo mitochondrial metabolic suppression in hibernation.

Acknowledgments

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