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# Tissue-specific depression of mitochondrial proton leak and substrate oxidation in hibernating arctic ground squirrels

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**Barger, Jamie L., Martin D. Brand, Brian M. Barnes, and Bert B. Boyer.** Tissue-specific depression of mitochondrial proton leak and substrate oxidation in hibernating arctic ground squirrels. *Am J Physiol Regul Integr Comp Physiol* 284: R1306–R1313, 2003. First published January 23, 2003; 10.1152/ajpregu.00579.2002.—A significant proportion of standard metabolic rate is devoted to driving mitochondrial proton leak, and this futile cycle may be a site of metabolic control during hibernation. To determine if the proton leak pathway is decreased during metabolic depression related to hibernation, mitochondria were isolated from liver and skeletal muscle of nonhibernating (active) and hibernating arctic ground squirrels (*Spermophilus parryii*). At an assay temperature of 37°C, state 3 and state 4 respiration rates and state 4 membrane potential were significantly depressed in liver mitochondria isolated from hibernators. In contrast, state 3 and state 4 respiration rates and membrane potentials were unchanged during hibernation in skeletal muscle mitochondria. The decrease in oxygen consumption of liver mitochondria was achieved by reduced activity of the set of reactions generating the proton gradient but not by a lowered proton permeability. These results suggest that mitochondrial proton conductance is unchanged during hibernation and that the reduced metabolism in hibernators is a partial consequence of tissue-specific depression of substrate oxidation.

hibernation; uncoupling; liver; skeletal muscle; metabolic depression

A CENTRAL QUESTION in mammalian hibernation is whether the low metabolic rates observed in hibernating animals are due to active suppression of metabolism or rather are a consequence of reduced enzyme activities at low body temperatures. In general, studies that have examined the effect of body temperature on whole animal metabolic rate support the temperature-dependent mechanism of metabolic suppression (20, 38). However, studies of isolated mitochondria have demonstrated that rates of oxygen consumption are reduced during hibernation and that this metabolic depression is independent of assay temperature (13, 19, 22, 25, 30, 32). Nevertheless, many of the studies in hibernators have only examined mitochondria isolated from liver, and when additional tissues have been investigated, results have been mixed. Hannon et al.

(22) found that respiration of skeletal muscle homogenates was depressed in hibernating arctic ground squirrels (*Spermophilus parryii*), whereas Brustovetsky et al. (11) found that heart and skeletal muscle mitochondrial respiration was increased in hibernating compared with active arctic ground squirrels. If metabolic depression is tissue specific, this may account for the discrepancy between whole animal and mitochondrial studies, as whole animal oxygen consumption is a composite of total metabolic activity and may fail to resolve mitochondrial activity among individual tissues.

When investigating metabolic depression in isolated mitochondria, two commonly measured parameters are state 3 and 4 respiration rates. State 3 respiration reflects oxygen consumption during ATP synthesis and is reduced during hibernation (13, 19, 25, 28, 32), possibly as a component of the decreased ATP turnover characteristic of many species during metabolic depression (21). State 4 (nonphosphorylating) oxygen consumption is indicative of proton leak across the mitochondrial inner membrane and is an inefficiency inherent to all mitochondria studied (6). Several studies report a decrease in state 4 respiration during hibernation in liver mitochondria (13, 17, 19, 30), which suggests that mitochondrial membranes are less permeable to protons. While this has been observed in other model systems (23), it is often incorrectly assumed that this is the case in hibernation. An alternative interpretation is that the force driving proton leak, the mitochondrial membrane potential ( $\Delta\psi_m$ ), is reduced during hibernation, and therefore decreased state 4 respiration may be a consequence of upstream metabolic control. Mechanistically, this can be achieved by decreasing the activity of the enzymes responsible for generating the  $\Delta\psi_m$ , including substrate translocases, dehydrogenases, and enzymes of the respiratory chain (7). Distinguishing which of these alternatives is responsible for metabolic depression requires parallel measurement of oxygen consumption  $\Delta\psi_m$  and may have important implications for the evolutionary conservation of metabolic depression across a wide array of species.

Therefore, a major goal of this investigation was to identify mechanisms responsible for the control of mi-

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tochondrial bioenergetics during mammalian hibernation. We studied mitochondria isolated from the arctic ground squirrel, which has a hibernating metabolic rate <1% of the nonhibernating metabolic rate when housed at temperatures near 0°C (14). We isolated mitochondria from liver and skeletal muscle because metabolic activity of these tissues constitutes 35–50% of standard metabolic rate (33), and the oxygen consumption driving proton leak in these tissues constitutes 15–20% of standard metabolic rate (36); therefore, proton leak in these tissues is a likely site for metabolic control during hibernation.

## METHODS

**Animals.** Adult arctic ground squirrels were trapped in the Alaska Range (64°N 146°W, elevation 850 m) in July and maintained at the University of Alaska Fairbanks. Animals were housed at  $5 \pm 2^\circ\text{C}$  with a 4:20-h light-dark photoperiod and were given Mazuri Rodent Chow (St. Louis, MO), sunflower seeds, carrots, apple slices, and water ad libitum. Animals were inspected twice daily, and wood shavings were placed on the dorsal surface of hibernating animals to assess the pattern of hibernation and arousal episodes. Tissues were collected from hibernators after no fewer than 5 days into at least the third torpor bout; active (nonhibernating) animals had not previously shown torpor as estimated by daily inspection.

Animals were always pair sampled (active and hibernating,  $n = 5$  animals/group) to minimize variation due to mitochondrial isolation techniques; all experiments were performed between 7 November and 22 December. Active animals were anesthetized with halothane (Halocarbon Products; North Augusta, SC) and rapidly decapitated. Rectal temperature ( $T_{\text{rect}}$ ) was measured in hibernators by inserting a thermocouple 2–3 cm into the rectum and allowing 1 min for the reading to stabilize; the average  $T_{\text{rect}}$  for hibernating animals was  $4.2 \pm 0.6^\circ\text{C}$  (range 2.1–5.7°C). Hibernators were euthanized by decapitation without anesthesia. Liver and gastrocnemius muscle (hereafter referred to as skeletal muscle) were rapidly dissected and transferred into ice-cold buffers for isolation of mitochondria.

All animal care and experimental protocols received approval from the University of Alaska Fairbanks Institutional Animal Care and Use Committee, which is fully compliant with the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings" (1).

**Isolation of mitochondria.** Isolation procedures were performed on ice, and all centrifuge spins were conducted at  $2 \pm 2^\circ\text{C}$ . Unless otherwise mentioned, all reagents were obtained from Sigma-Aldrich (St. Louis, MO). Liver mitochondria were isolated by disrupting tissue using a Dounce homogenizer with a loose-fitting pestle in a buffer containing 250 mM sucrose, 5 mM Trizma, and 2 mM EGTA. The homogenate was spun at 490  $g$  for 10 min, and the resulting supernatant was subjected to a high-speed spin cycle (10,550  $g$  for 10 min). The supernatant was poured off, and lipid remaining on the inner surface of the tube was removed using a paper tissue. The crude mitochondrial pellet was resuspended in homogenization buffer containing 0.5% fatty acid-free BSA (Intergen; Purchase, NY) to chelate endogenous fatty acids and subjected to an additional high-speed spin cycle. The resulting pellet was resuspended in homogenization buffer (–BSA) and subjected to another high-speed spin cycle. The final pellet was resuspended in a minimal volume of homogenization buffer (–BSA) and stored on ice.

Skeletal muscle was trimmed of fat and connective tissue, finely minced with a razor blade on a glass plate over ice, and transferred to a beaker containing ice-cold wash buffer (100 mM KCl, 50 mM Tris·HCl, and 2 mM EGTA). The excess buffer was poured off and replaced with additional buffer; this process was repeated an additional five times to remove any hair, fat, and connective tissue from the preparation. After the final wash, excess buffer was poured off and replaced with a digestion buffer (10 ml/g tissue) containing 100 mM KCl, 50 mM Tris·HCl, 2 mM EGTA, 0.5% fatty acid-free BSA, 5 mM  $\text{MgCl}_2$ , 1 mM ATP, and nagarse (18.7 U/g tissue). This mixture was kept on ice for 10 min with occasional stirring and then briefly homogenized with a polytron ( $2 \times 10$  s). After an additional 10 min with occasional stirring on ice, the homogenate was spun at 490  $g$  for 10 min. The supernatant was filtered through three layers of gauze and spun at 10,500  $g$  for 10 min. The crude mitochondrial pellet was resuspended in excess wash buffer and subjected to an additional high-speed spin cycle (10,500  $g$  for 10 min). The final pellet was resuspended in a minimal volume of wash buffer and kept on ice.

Protein concentration of all mitochondria preparations was determined in duplicate by the bicinchoninic acid assay (Pierce Chemical BCA Protein Assay Kit; Rockford, IL) with BSA as the standard. Integrity of isolated mitochondria was estimated by calculating the respiratory control ratio (RCR) for each preparation (state 3  $\div$  state 4 respiration rates).

**Measurement of mitochondrial bioenergetics.** All assays were performed at 37°C in 3 ml of assay buffer containing mitochondria (liver = 1 mg protein/ml, skeletal muscle = 0.5 mg protein/ml), 120 mM KCl, 5 mM  $\text{KH}_2\text{PO}_4$ , 3 mM HEPES, 1 mM EGTA, and 0.5% fatty acid-free BSA, pH 7.2. We chose this temperature because it is physiological for active animals and because tissues of hibernators are routinely exposed to this temperature during arousal from hibernation, whereas mitochondria from active animals are never exposed to lower temperatures. A dual-channel chart recorder (Kipp and Zonen) was used to record simultaneous measurements of oxygen consumption and membrane potential. Oxygen consumption was measured using a Rank Brothers Model 10 oxygen electrode (Cambridge, UK) assuming 406 nmol O/ml buffer (31). The  $\Delta\psi_m$  (mV) was estimated by uptake of the lipophilic cation triphenylmethylphosphonium (TPMP) using the following equation

$$\Delta\psi_m = 61.5 \log \left\{ \frac{([\text{TPMP}] \text{ added} - [\text{TPMP}] \text{ external}) \times \text{TPMP binding correction}}{0.001 \times [\text{protein}] \times [\text{TPMP}] \text{ external}} \right\}$$

To estimate the concentration of TPMP in the assay buffer ([TPMP] external), a standard curve of TPMP concentration and chart recorder distance was generated with incremental additions of TPMP (1, 2, 3, 4, 5  $\mu\text{M}$ ) for each assay. Electrode drift was corrected after each run by addition of 2  $\mu\text{M}$  carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazide (FCCP). Mitochondrial TPMP binding corrections were 0.4 and 0.35 for liver and skeletal muscle, respectively (35). Using a constant TPMP binding correction factor for active and hibernating animals is unlikely to influence our estimation of  $\Delta\psi_m$  for at least two reasons:  $\Delta\psi_m$  is a logarithmic function of internal and external TPMP concentrations (above), and therefore large changes in TPMP binding are required to effect a modest change in  $\Delta\psi_m$  (e.g., a 2-fold increase in TPMP binding yields a 20% increase in  $\Delta\psi_m$ ); second, studies are equivocal as to whether mitochondrial ultrastructure increases (27), decreases (12), or does not change (28) during hibernation.

Table 1. *Bioenergetic properties of liver and skeletal muscle mitochondria*

	Liver		Skeletal Muscle	
	Active	Hibernating	Active	Hibernating
State 3 $\Delta\psi_m$	134.7 $\pm$ 2.6	124.0 $\pm$ 2.3	135.8 $\pm$ 4.2	128.8 $\pm$ 2.7
State 4 $\Delta\psi_m$	196.0 $\pm$ 1.9	181.6 $\pm$ 3.5*	195.5 $\pm$ 2.9	192.6 $\pm$ 3.1
State 3 respiration	253.1 $\pm$ 18.7	76.9 $\pm$ 10.4†	602.3 $\pm$ 36.7	481.9 $\pm$ 25.8
State 4 respiration	33.0 $\pm$ 2.9	19.4 $\pm$ 2.0*	107.9 $\pm$ 5.2	95.6 $\pm$ 7.3
Respiratory control ratio	7.8 $\pm$ 0.5	4.3 $\pm$ 0.9	5.6 $\pm$ 0.2	5.2 $\pm$ 0.2

Values are means  $\pm$  SE. Mitochondrial membrane potentials ( $\Delta\psi_m$ ) are shown in mV; respiratory rates are shown as nmol O $\cdot$ min $^{-1}$ ·mg mitochondrial protein $^{-1}$  (see METHODS for specific assay conditions). Respiratory control ratios were calculated as state 3 respiration  $\div$  state 4 respiration. Significant differences between active and hibernating groups: \* $P$  < 0.05, † $P$  < 0.001.

The kinetics of proton leak and substrate oxidation were assayed in triplicate and duplicate, respectively, for each mitochondrial preparation (5). The kinetics of proton leak were measured by titrating state 4 respiration with malonate, a competitive inhibitor of succinate (liver = 0, 0.5, 1, 1.5, 2, 2.5 mM malonate; muscle = 0, 0.3, 0.6, 1, 1.3 mM malonate). The kinetics of substrate oxidation were estimated by measuring the stimulation of the respiratory chain in response to decreases in  $\Delta\psi_m$  induced in the transition from state 4 to state 3 conditions. State 3 conditions were established by incubating mitochondria in the presence of 5  $\mu$ M rotenone (to inhibit complex I oxidation of endogenous NADH), 80 ng/ml nigericin (to set  $\Delta$ pH to zero), 250  $\mu$ M ADP, and 5 mM succinate. State 4 conditions were obtained by incubating mitochondria in 5  $\mu$ M rotenone, 80 ng/ml nigericin, 5 mM succinate, and 1  $\mu$ g/ml oligomycin, a specific inhibitor of the F $_0$ F $_1$  ATP synthase.

**Statistics.** Mean values for active and hibernating groups were compared using Student's *t*-tests; adjustment for post hoc multiple comparisons was performed using Holm's step-down modification of Bonferroni (24).

## RESULTS

**Bioenergetics of liver mitochondria.** The RCRs of liver mitochondria were lower in hibernating animals (Table 1), but this was not statistically significant. A decreased RCR can arise if state 4 respiration is increased due to physical disruption of mitochondrial membranes during isolation. However, state 4 respiration was decreased in hibernators, and therefore the low RCR is likely due to depressed state 3 respiration, not physically damaged mitochondria.

Representative traces of the proton leak assay in liver mitochondria for an active and a hibernating arctic ground squirrel are shown in Fig. 1; these plots show that mitochondria isolated from hibernating animals had lower rates of state 4 respiration and were unable to achieve the high  $\Delta\psi_m$  of mitochondria isolated from active squirrels. These traces were used to determine the kinetics of the proton leak system that

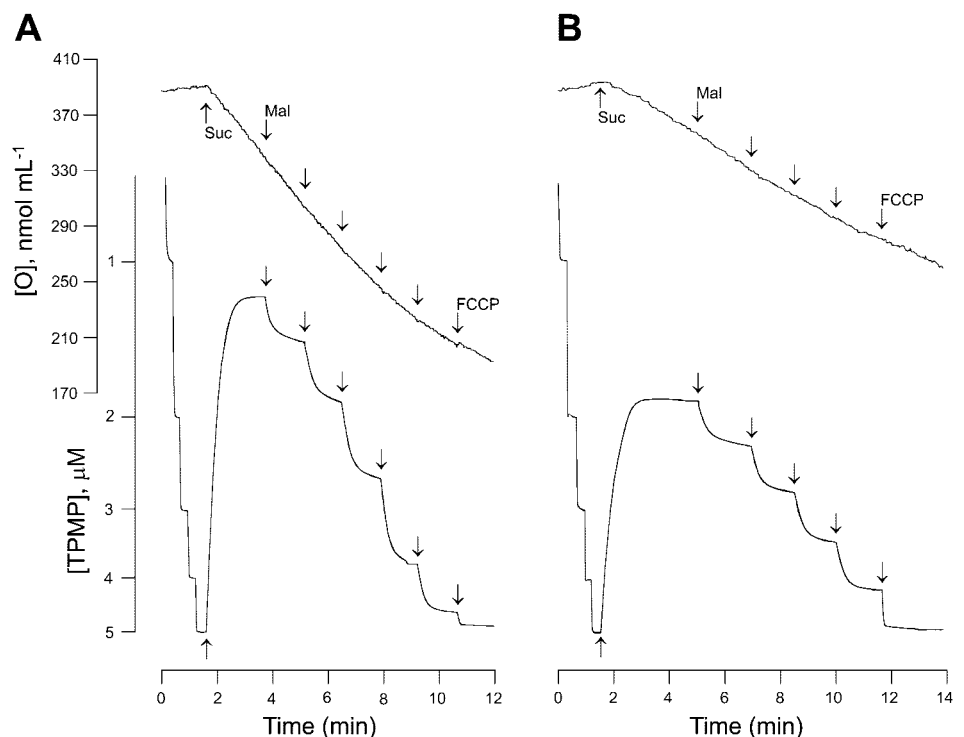


Fig. 1. Representative traces showing the relationship between the concentrations of oxygen (top traces) and triphenylmethylphosphonium (TPMP; bottom traces) in assay buffer containing mitochondria isolated from the livers of an active (A) and a hibernating (B) arctic ground squirrel sampled on the same day. Mitochondria were incubated in assay buffer, rotenone, oligomycin, and nigericin for at least 1 min before TPMP additions. Upward arrows designate addition of succinate (Suc), and downward arrows designate the successive additions of malonate (Mal) or the final addition of a chemical uncoupler [carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; FCCP]; arrows on the TPMP traces correspond with those on the oxygen traces. Mitochondrial membrane potential is calculated from, and is an inverse function of, the concentration of TPMP in assay buffer, such that a low concentration of TPMP in the buffer reflects a high  $\Delta\psi_m$ . See METHODS for details.

are summarized in Fig. 2A. Although state 4 respiration and  $\Delta\psi_m$  were significantly different between active and hibernating groups (Table 1), the curves overlapped such that the rate of proton leak was not significantly different at any common values of  $\Delta\psi_m$  (range ~135–180 mV). This suggests that the proton permeability of the mitochondrial membrane was unchanged during hibernation, which was corroborated by estimating proton conductance (proton leak rate per mV) at a  $\Delta\psi_m$  of 180 mV, the highest potential achieved by both groups. Proton conductance was not significantly different between groups, as evidenced by an overlap in 95% confidence intervals (Fig. 3).

State 3 respiration rate, but not  $\Delta\psi_m$ , was significantly depressed in hibernators (Table 1). Plotting mitochondrial respiration and membrane potential for both state 3 and 4 conditions describes the ability of the substrate oxidation system to respond to changes in

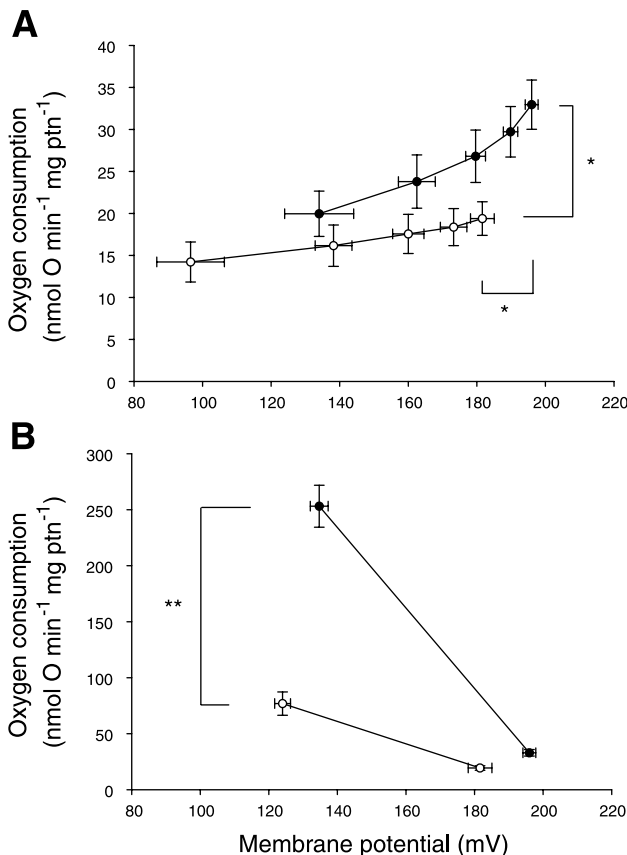


Fig. 2. Kinetic response of the proton leak (A) and substrate oxidation (B) systems to  $\Delta\psi_m$  in liver mitochondria isolated from active and hibernating arctic ground squirrels. Filled symbols, active animals; open symbols, hibernating animals. A: maximal state 4 conditions are shown as the top right-most points for each plot; respiration (proton leak rate) and  $\Delta\psi_m$  were significantly lower in hibernators ( $*P < 0.05$ ). Across the range of  $\Delta\psi_m$  common to both treatments (~135–180 mV), rates of proton leak did not differ between groups based on overlapping SDs (not shown). B: state 4 and state 3 conditions are shown as the bottom right-most and top left-most points, respectively; state 3 respiration was significantly lower in hibernators ( $**P < 0.001$ ); lines designating a significant reduction of state 4  $\Delta\psi_m$  and respiration are omitted for clarity (see A). Values are means  $\pm$  SE;  $n = 5$  animals/treatment.

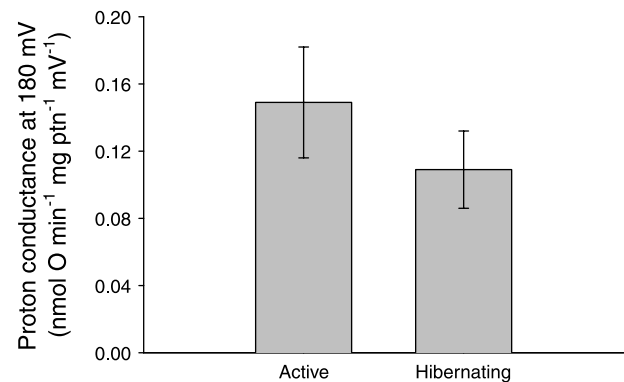


Fig. 3. Estimated proton conductance of mitochondria isolated from liver of arctic ground squirrels at a  $\Delta\psi_m$  of 180 mV (the highest  $\Delta\psi_m$  common to both active and hibernating groups). Proton conductance, defined as proton leak rate per mV, was calculated as proton leak rate  $\div$   $\Delta\psi_m$  and was not significantly different between active and hibernating groups. Values are means  $\pm$  95% confidence intervals.

$\Delta\psi_m$ . Across the range of  $\Delta\psi_m$  common to both treatments (~135–180 mV), the activity of the substrate oxidation system was approximately one-fourth that of the rate in the active animals at a given value of  $\Delta\psi_m$  (Fig. 2B).

**Bioenergetics of skeletal muscle mitochondria.** In mitochondria isolated from skeletal muscle, state 4  $\Delta\psi_m$  and respiration were not significantly different between hibernating and active groups (Table 1); similarly, the kinetics of proton leak were virtually identical (Fig. 4A). Although state 3 respiration of skeletal muscle mitochondria was decreased in hibernators by ~20% (compared with active squirrels), there was no statistically significant difference in state 3 respiration or  $\Delta\psi_m$  between groups (Table 1). In contrast to the pattern seen in liver mitochondria, the activity of the substrate oxidation system in skeletal muscle mitochondria was unchanged in hibernators; across the range of membrane potentials common to both groups, oxygen consumption was similar for both groups (Fig. 4B).

## DISCUSSION

Proton leak across the mitochondrial inner membrane accounts for 20–30% of standard metabolic rate (6) and represents a significant inefficiency because energy released during substrate oxidation is not conserved as ATP. During hibernation, arctic ground squirrels have a metabolic rate ~1% of the resting metabolic rate in nonhibernating animals, and therefore control of proton leak is likely during hibernation. Our study demonstrates that in arctic ground squirrels, the rate of proton leak is actively depressed during hibernation and that this depression persists at high (37°C) assay temperatures. However, the reduced proton leak was not achieved by decreasing membrane permeability. Instead, the decreased rate of proton leak was controlled by an upstream reduction in the substrate oxidation system, as demonstrated in skeletal muscle of frogs (*Rana temporaria*) held under hypoxia (39) and in snail (*Helix aspersa*) hepatopan-

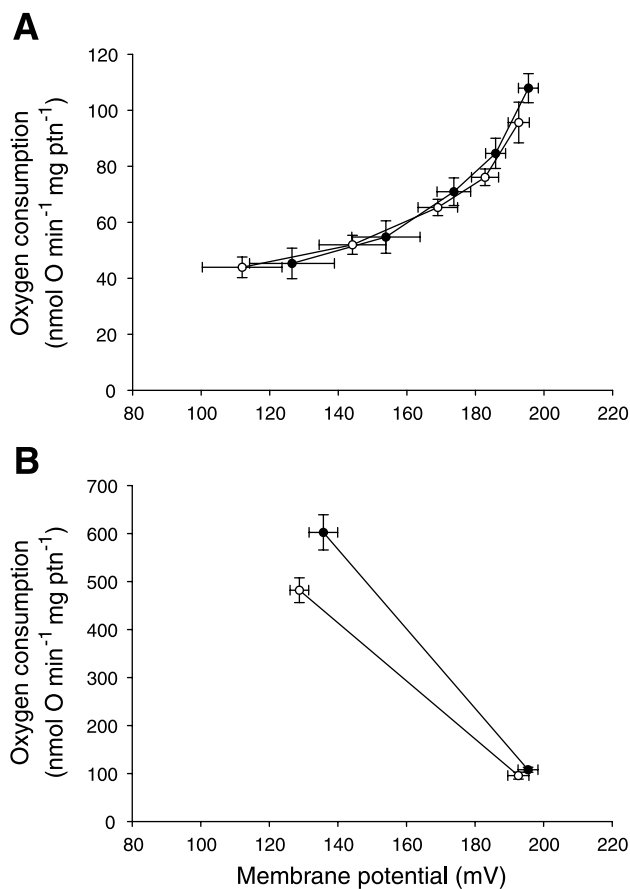


Fig. 4. Kinetic response of the proton leak (A) and substrate oxidation (B) systems to  $\Delta\psi_m$  in skeletal muscle mitochondria isolated from active and hibernating arctic ground squirrels. Filled symbols, active animals; open symbols, hibernating animals. A: maximal state 4 conditions are shown as the top right-most points for each plot; state 4  $\Delta\psi_m$  and respiration were not different between treatments. B: state 4 and state 3 conditions are shown as the bottom right-most and top left-most point, respectively. Values are means  $\pm$  SE;  $n = 5$  animals/treatment.

creas cells during estivation (2). In arctic ground squirrels, the reduction of proton cycling was tissue specific: proton leak was depressed in liver mitochondria of hibernators, but mitochondria isolated from skeletal muscle from hibernating and active animals exhibited nearly identical bioenergetic profiles.

**Kinetics of proton leak.** Mitochondrial respiration under nonphosphorylating (state 4) conditions is used to drive the leak of protons across the mitochondrial inner membrane. Results from studies of mitochondria isolated from the liver of hibernating Richardson's (30) and arctic ground squirrels (13, 17) showed lower rates of state 4 respiration compared with nonhibernating animals. This depression was independent of assay temperature and was reversed during arousal from hibernation (17, 30). In contrast, Martin et al. (28) found no differences in state 4 respiration in liver mitochondria isolated from hibernating, arousing, or summer-active golden-mantled ground squirrels. The results from the present study are in agreement with the former studies: during hibernation, state 4 respi-

ration was decreased by 41% in mitochondria isolated from liver and assayed at 37°C (Table 1). Procedural differences may account for the discrepancy between our and previous (28) studies: magnesium inhibits succinate dehydrogenase in rat liver (26, 29) and skeletal muscle (15) mitochondria, and the inclusion of magnesium in the assay buffer (28) may have masked differences in state 4 respiration between hibernating and active animals. This possibility is supported by the finding that the average state 4 respiration of both active and hibernating liver mitochondria in golden-mantled ground squirrels (28) was less than that measured in hibernators in this study and in Richardson's ground squirrels (30).

State 4 respiration is a crude indicator of proton leak, and parallel measurements of oxygen consumption and membrane potential are required to determine if a change in the magnitude of proton leak is due to a change in membrane proton permeability per se. Proton permeability can be determined by estimating the membrane proton conductance, defined as the rate of proton leak at a common value of  $\Delta\psi_m$ . Decreased mitochondrial membrane proton permeability has been observed experimentally and accounts for ~50% of the decrease in respiration rate between hyperthyroid and euthyroid rat hepatocytes (23). In addition, the difference in mitochondrial respiration between endotherms and ectotherms is also attributable to decreased proton conductance (9, 39). A decrease in proton permeability has not been demonstrated, however, in organisms that depress metabolic rate: state 4 respiration was decreased in skeletal muscle mitochondria isolated from frogs held under hypoxia vs. normoxia, but rates of oxygen consumption did not differ between groups at common values of  $\Delta\psi_m$  (39). A similar study demonstrated that proton permeability is unchanged in hepatopancreas cells isolated from metabolically depressed snails (2). The same conclusion was found for liver mitochondria in the present study: although state 4 respiration was decreased in hibernators, the rate of proton leak did not differ for values of  $\Delta\psi_m$  that were attained by mitochondria in both groups. Specifically, estimated proton conductance did not differ between hibernating and active groups at 180 mV, the highest  $\Delta\psi_m$  that was common to both treatments (Fig. 3). It is possible that this more stringent analysis underestimates the actual response, i.e., fails to identify a decrease in conductance, and therefore some of the decrease in oxygen consumption may be due to a modest depression of proton leak that could not be resolved using our techniques. However, the finding that the maximal (state 4)  $\Delta\psi_m$  achieved by hibernators was significantly lower than that of the active animals (Fig. 2A) corroborates the conclusion that proton permeability is not decreased in liver mitochondria during hibernation.

There are few studies of the effect of hibernation on mitochondrial bioenergetics in tissues other than liver. Brustovetsky et al. (11) reported that heart and skeletal muscle mitochondria isolated from hibernating arctic ground squirrels had elevated levels of state 4

respiration compared with nonhibernators. This increase may have been due to fatty acid-induced uncoupling (40), as rates were similar between groups when mitochondria were assayed in the presence of BSA. The present study reports similar findings in skeletal muscle: state 4 respiration and  $\Delta\psi_m$  were identical between hibernators and active squirrels, and the kinetic response of the proton leak was identical across all values of  $\Delta\psi_m$  (Fig. 4A).

*Kinetics of substrate oxidation.* An alternative means of decreasing proton leak (other than decreasing membrane permeability) is by controlling the set of reactions that generate the proton gradient, namely the enzymes of substrate oxidation. A number of studies suggest that substrate oxidation is decreased in liver mitochondria isolated from hibernating arctic ground squirrels (10, 13, 17), but membrane potential was not measured, and it is formally possible that the decreased respiration in these studies was attributable to decreased proton permeability.

The kinetics of substrate oxidation are typically measured by titrating state 4 respiration with a chemical uncoupler; the resulting drop in membrane potential causes a stimulation of mitochondrial respiration, and the magnitude of this increase reflects the activity of the substrate oxidation subsystem (4). This response can also be estimated by plotting respiration rate against  $\Delta\psi_m$  measured under state 3 and state 4 conditions. The line generated between these two points estimates the kinetic response of the substrate oxidation system to changes in  $\Delta\psi_m$ . In liver mitochondria isolated from hibernating squirrels, respiration rate was approximately one-fourth that of active squirrels across the range of  $\Delta\psi_m$  between state 4 and state 3 conditions (Fig. 2B). In the absence of a change in membrane permeability (above), these data suggest that control of substrate oxidation reactions is the means by which mitochondrial proton cycling is reduced during hibernation in arctic ground squirrels. Interestingly, decreased activity of the enzymes of substrate oxidation appears to be the predominant mechanism by which proton cycling is reduced during metabolic depression in general, as this pattern was observed in mitochondria isolated from skeletal muscle of frogs housed under hypoxia (39) and snail hepatopancreas cells (2) during estivation. Although the data from this study do not reveal which components of the substrate oxidation module are depressed, others have suggested that control of respiratory activity of liver mitochondria during hibernation may be attributable to a decreased activity of succinate dehydrogenase (16, 17, 25, 30), a decreased rate of electron transfer in the respiratory chain (10), or altered membrane fluidity due to a lower activity of phospholipase A<sub>2</sub> (13).

In contrast to the pattern seen in liver, the kinetics of substrate oxidation do not appear to be decreased during hibernation in mitochondria isolated from skeletal muscle: respiration rate and  $\Delta\psi_m$  were similar under both state 3 and 4 conditions (Fig. 4B), and rates of FCCP-uncoupled respiration did not differ between active and hibernating groups (data not shown). The

reason for this tissue-specific difference is not known, although the substrate oxidation module tends to be more active in skeletal muscle than in liver (compare Figs. 2B and 4B, and see Ref. 35) and perhaps is less able to be controlled at the mitochondrial level. Alternatively, it is possible that substrate oxidation could be controlled in vivo at the organ level via decreased perfusion of skeletal muscle during hibernation, which would putatively limit substrate supply. Measurement of mitochondrial activity in intact skeletal muscle (34) might resolve this issue.

*The hibernating mitochondrion.* This is the first study to investigate the kinetics of mitochondrial proton leak in a hibernating mammal and shows that futile proton cycling during hibernation is not decreased by altering the membrane proton permeability. Although the maximal rate of proton leak (state 4 respiration) was significantly reduced by 41% during hibernation in liver mitochondria (Fig. 2A), concurrent measurement of  $\Delta\psi_m$  revealed that proton conductance was unchanged in hibernation (Fig. 3). This distinction highlights the pitfall of estimating proton leak by measuring state 4 respiration alone: the rate of proton leak exhibits steep dependence on  $\Delta\psi_m$  at high potentials, and therefore minor changes in the  $\Delta\psi_m$  can substantially affect the magnitude of the proton leak (8). In liver mitochondria, state 4  $\Delta\psi_m$  was significantly reduced by 7% in hibernators, and therefore the reduced state 4 respiration was a consequence of the decreased force driving the proton leak. Mechanistically, the  $\Delta\psi_m$  can be regulated by inhibiting the activity of the respiratory chain, and this appears to be the case in hibernation; the activity of the respiratory chain in liver mitochondria was reduced by ~75% across the range of  $\Delta\psi_m$  values common to both groups (Fig. 2B).

The reduced body temperature of hibernators undoubtedly influences mitochondrial enzyme activity, and active depression of substrate oxidation probably accounts for a comparatively small proportion of metabolic depression in vivo. Nevertheless, the mechanism underlying mitochondrial metabolic depression observed in this study is conserved across diverse taxa (39), including one species in which metabolic depression occurs in the absence of changes in temperature (2). This provides strong evidence to support the hypothesis that metabolic processes are actively controlled during hibernation and are not solely a function of reduced enzyme activity at low body temperatures. It will be interesting to see if depression of substrate oxidation is restricted to metabolic depression associated with estivation/hibernation or instead is a more widespread phenomenon common to an array of species in response to decreased energy availability (41).

### Perspectives

Active depression of metabolism permits survival during periods of energy limitation and is a widespread phenomenon that involves coordinated regulation of ATP supply and demand (21). This can be achieved through decreasing energy-expensive processes such

as protein synthesis (18), maintenance of ion gradients (3), and proton leak (2, 39; this study). To date, all studies of mitochondrial proton cycling during metabolic depression have revealed that decreased proton leak is achieved by decreasing its driving force (substrate oxidation reactions) rather than by decreasing the permeability of the membrane to protons. This similar pattern of control across a diverse range of taxa suggests an evolutionarily conserved mechanism for metabolic control at the mitochondrial level, although the mechanism is unknown. In addition, this study found that control is tissue specific, which may account for discrepancies between studies of isolated mitochondria and whole animal metabolism.

Surprisingly, organisms devote a significant amount of respiration toward driving proton leak even during metabolic depression, suggesting that proton leak serves a critical function that must be retained despite its cost. Proton leak may ameliorate the formation of reactive oxygen species (ROS) (37), and it is tempting to speculate that the maintenance of proton cycling in skeletal muscle represents a mechanism to prevent oxidative damage during ischemia-reperfusion injury during arousal from hibernation. The fact that inhibition of mitochondrial respiration in other tissues is reversed during arousal from hibernation also supports the hypothesis that proton leak may protect against ROS generation during tissue reperfusion. While there is scant evidence that mitochondrial proton leak ameliorates oxidative damage in vivo, hibernating animals may be a useful model system toward this end.

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