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HIGHLIGHTED TOPIC | *A Physiological Systems Approach to Human and Mammalian Thermoregulation*

Regulation of UCP1 and UCP3 in arctic ground squirrels and relation with mitochondrial proton leak

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Barger, Jamie L., Brian M. Barnes, and Bert B. Boyer. Regulation of UCP1 and UCP3 in arctic ground squirrels and relation with mitochondrial proton leak. *J Appl Physiol* 101: 339–347, 2006; doi:10.1152/japplphysiol.01260.2005.—Uncoupling protein (UCP) 1 (UCP1) catalyzes a proton leak in brown adipose tissue (BAT) mitochondria that results in nonshivering thermogenesis (NST), but the extent to which UCP homologs mediate NST in other tissues is controversial. To clarify the role of UCP3 in mediating NST in a hibernating species, we measured *Ucp3* expression in skeletal muscle of arctic ground squirrels in one of three activity states (not hibernating, not hibernating and fasted for 48 h, or hibernating) and housed at 5°C or −10°C. We then compared *Ucp3* mRNA levels in skeletal muscle with *Ucp1* mRNA and UCP1 protein levels in BAT in the same animals. *Ucp1* mRNA and UCP1 protein levels were increased on cold exposure and decreased with fasting, with the highest UCP1 levels in thermogenic hibernators. In contrast, *Ucp3* mRNA levels were not affected by temperature but were increased 10-fold during fasting and >3-fold during hibernation. UCP3 protein levels were increased nearly fivefold in skeletal muscle mitochondria isolated from fasted squirrels compared with nonhibernators, but proton leak kinetics in the presence of BSA were unchanged. Proton leak in BAT mitochondria also did not differ between fed and fasted animals but did show classical inhibition by the purine nucleotide GDP. Levels of nonesterified fatty acids were highest during hibernation, and tissue temperatures during hibernation were related to *Ucp1,* but not *Ucp3,* expression. Taken together, these results do not support a role for UCP3 as a physiologically relevant mediator of NST in muscle.

hibernation; nonshivering thermogenesis; fasting; fatty acid; obesity

NONSHIVERING THERMOGENESIS (NST) is classically associated with the presence of uncoupling protein (UCP) 1 (UCP1) in brown adipose tissue (BAT). UCP1 is a transmembrane protein located in the mitochondrial inner membrane, which, on activation by the sympathetic nervous system, allows for protons to reenter the mitochondrial matrix without passing through ATP synthase. Heat is generated, because the energy released in substrate oxidation is not conserved as ATP (40). Although there are reports of NST in species lacking BAT (4, 21, 26, 43), the underlying molecular mechanisms have remained elusive.

Four genes have been identified that encode proteins homologous to UCP1 and may provide a possible explanation for NST in tissues other than BAT: UCP2 is expressed in a wide array of tissues and is 55% similar to UCP1 (22); UCP3 is expressed predominantly in skeletal muscle and BAT and is 57% similar to UCP1 (7, 50); UCP4 and brain mitochondrial carrier protein (BMCP1) are expressed predominantly in neural tissues (36, 44) and are 29% and 34% similar to UCP1, respectively. In addition to sequence homology, early reports suggested that the expression pattern of these genes was consistent with a role in NST: thyroid hormone, cold exposure, and high-fat diet were associated with increased levels of *Ucp* homolog mRNA (24, 33, 34, 53). Moreover, the decreased membrane potential in yeast transfected with human *Ucp3* suggests that UCP3 may catalyze mitochondrial uncoupling (27). Finally, ATP synthesis in muscle is increased in *Ucp3*-knockout mice (20), and muscle temperature is elevated in mice overexpressing human *Ucp3* (19), consistent with a role in energy metabolism.

However, other data suggest that the UCP homologs do not participate in NST in vivo: the decreased membrane potential in yeast expressing human *Ucp3* and the increased uncoupled respiration in transgenic mice are likely artifacts of supraphysiological UCP3 levels (16, 28). Furthermore, *Ucp3*$^{-/-}$ mice are not obese, sensitive to cold, or hypothermic, suggesting that UCP3 does not exert a significant effect on whole animal metabolism (25, 51). Finally, fasting decreases BAT *Ucp1* mRNA and UCP1 (18, 39) yet, paradoxically, increases *Ucp3* expression in skeletal muscle (24) and heart (49).

The vast majority of studies investigating the function of the newly identified UCP homologs have utilized mice and rats; however, species with a greater capacity for metabolic heat production may be ideally suited for testing the hypothesis that UCP homologs mediate NST. The arctic ground squirrel (*Spermophilus parryii*) is able to lower its body temperature to −2.9°C during hibernation and then rewarm to 37°C during periodic arousal episodes (3). Consistent with a thermoregulatory role, levels of skeletal muscle *Ucp3* mRNA were reported to be increased during hibernation in this species (8). An alternative explanation for the observed increase in *Ucp3* expression during hibernation is that the fasted state accompanying hibernation regulates *Ucp3* expression. UCP3 has been proposed to coordinate the metabolic shift toward fatty acid oxidation (29), and, according to this model, the elevated levels of *Ucp3* mRNA during hibernation may be attributable to fasting, rather than a need for NST.

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To distinguish between the effects of thermogenic demand and fasting, we measured levels of *Ucp1* mRNA and UCP1 protein in BAT, levels of skeletal muscle *Ucp3* mRNA, and serum levels of nonesterified fatty acids (NEFA) in arctic ground squirrels in one of three activity states (not hibernating, not hibernating and fasted for 48 h, or hibernating) and housed at 5°C or −10°C. During housing at 5°C, NST is minimal in nonhibernating arctic ground squirrels and is essentially absent in hibernators, because body temperature remains at equilibrium with the ambient temperature. In contrast, NST will be near maximal levels in animals housed at −10°C to defend a high body temperature relative to the environment. If a gene (or its protein) contributes to NST, its abundance should increase when the animal is thermogenic but decrease during fasting. We also measured proton leak kinetics in mitochondria isolated from fed and fasted nonhibernators to determine whether the rate of proton leak correlates with changes in *Ucp* expression and UCP protein abundance. Finally, we measured the temperature of several tissues in a subset of hibernators housed at −10°C to determine whether increased *Ucp* expression was manifested as an increase in tissue temperature.

METHODS

Experimental design. Arctic ground squirrels were captured in the Alaska Range (64°N, elevation 1,200 m) during late July and transported to the University of Alaska Fairbanks. The animals were housed for 1 mo at 15°C with a photoperiod tracking natural conditions at the site of capture and then transferred to environmental chambers with a 4:20-h light-dark photoperiod and kept at 5°C or −10°C; these temperatures correspond to the lower end of the thermoneutral zone (45) and a typical winter burrow temperature (13) for this species, respectively. Mazuri rodent chow (PMI Nutrition, St. Louis, MO), sunflower seeds, carrots, and apple slices were available ad libitum for all animals; water was provided to animals housed at 5°C. All animal care and experimental protocols were approved by 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).

The animals were observed twice daily; on the 1st day of a hibernation bout (determined by posture and reduced respiration), wood shavings were applied to the dorsal surface of a squirrel. The persistence of wood shavings on the animal was used as the criterion for maintenance of torpor. Ground squirrels were included in the hibernating groups (HIB, $n = 8$ at 5°C and 11 at −10°C) when they had remained torpid for ≥5 days after at least three bouts of torpor. Active squirrels (ACT, $n = 5$ at 5°C and 9 at −10°C) consisted of animals that had not shown torpor according to the above criterion. An additional group of nonhibernating squirrels at each temperature was subjected to a 48-h fast (FAST, $n = 4$ each at 5°C and −10°C). All animals were sampled between 12 November and 2 February. To control for possible seasonal variation, ACT and HIB squirrels were pair-sampled; FAST squirrels were collected at three time points with ACT and HIB squirrels. Tissues from these animals were used for determination of levels of *Ucp1* mRNA, UCP1 protein, *Ucp3* mRNA, and NEFA. In seven squirrels hibernating at −10°C, a thermocouple was used to measure the temperature of several tissues immediately after they were exposed but before the tissue was excised from the animal.

A second set of experiments sought to determine whether physiological changes in UCP1 and UCP3 protein levels are accompanied by changes in mitochondrial proton leak. Arctic ground squirrels that were housed at 5°C but had not hibernated were randomly assigned to a fed ($n = 5$) or fasted ($n = 4$) treatment group; feeding regimens were the same as those described above. Tissues were collected for isolation of mitochondria and were, in turn, used for measurement of UCP1 and UCP3 protein levels as well as mitochondrial proton leak. All animals in this second set of experiments were sampled between 22 February and 28 March.

Tissue collection. Active (ACT and FAST) animals were lightly anesthetized with halothane (Halocarbon Products, North Augusta, SC), and a thermocouple was inserted ~2–3 cm into the rectum for measurement of rectal temperature (T_{re}), which was recorded after 1 min was allowed for the reading to stabilize; active animals with T_{re} <35°C were not used in this study. Blood was collected by cardiac puncture, and serum was stored at −70°C for analysis of NEFA using a commercially available kit (Wako Chemicals, Richmond, VA). After blood collection, active animals were euthanized with an overdose of pentobarbital sodium. Hibernating animals were removed from their cage, T_{re} was measured as described above, and blood was collected by cardiac puncture within 5 min; hibernating animals were then euthanized by decapitation. Axillary BAT and gastrocnemius muscle (hereafter referred to as skeletal muscle) were collected within 5 min, flash frozen in liquid nitrogen, and stored at −70°C. When needed, additional quantities of BAT and skeletal muscle were excised and transferred to ice-cold buffers for isolation of mitochondria.

Ucp mRNA levels. mRNA levels were quantified by Northern analysis as described previously (8). *Ucp1* mRNA was detected using a highly conserved 27-bp oligonucleotide probe (12), and *Ucp3* mRNA was detected using a 29-bp oligonucleotide probe (5′-CCT-TCCTCCCTGGCGATGGTTCTGTAGGC-3′) specific to arctic ground squirrel *Ucp3* (unpublished observations). Blots were later reprobed for 18S rRNA to adjust for variations in RNA loading. All autoradiograph exposures were maintained within the linear range of the film, and quantification of *Ucp* mRNA levels was determined by densitometric analysis of autoradiographs.

UCP protein abundance. UCP1 protein was detected in BAT homogenates and isolated mitochondria by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). BAT homogenates were prepared in ice-cold buffer (50 mM PIPES, 25 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM PMSF) with a protease inhibitor cocktail (1 mM benzamidine, 0.1 mM phenanthroline, and aprotonin, leupeptin, and pepstatin A at 1 mg/ml each) added immediately before homogenization at a concentration of 0.1 ml/10 ml of buffer. Dithiothreitol (0.1 mM) was added to the final homogenate. Total protein concentration was determined using the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL), and homogenates were stored at −70°C. Isolation of BAT mitochondria is described below.

BAT homogenates and previously frozen mitochondria (5 μg of total and mitochondrial protein, respectively) were fractionated in 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. UCP1 was detected with rabbit anti-rat UCP1 serum as described elsewhere (9). To estimate the absolute concentration of UCP1, at least four known concentrations of murine UCP1 protein purified from *Escherichia coli* inclusion bodies (46) were included in each blot. The abundance of arctic ground squirrel UCP1 was estimated by interpolation of the linear regression between the known UCP1 concentration and subsequent densitometric units.

We used freshly isolated skeletal muscle mitochondria (see below) for determination of UCP3 protein levels. Mitochondrial protein (25 μg) was separated by SDS-PAGE as described for UCP1, except the blots were hybridized for 12 h at 5°C with an antibody specific to amino acids 295–308 of mouse and rat UCP3 (Chemicon, Temecula, CA) at a dilution of 1:1,000. Under these conditions, arctic ground squirrel UCP3 was observed at the same molecular size as a positive control (~34 kDa in mouse heart mitochondrial protein), and no hybridization was observed with a negative control (mitochondrial protein from $Ucp3^{-/-}$ mice). UCP3 levels were quantified by interpolation from known concentrations of human UCP3 obtained from *E. coli* inclusion bodies (28).

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Isolation of mitochondria. All procedures were performed on ice, and all centrifuge spins were conducted at 2 ± 2°C. Unless otherwise noted, all reagents were obtained from Sigma-Aldrich (St. Louis, MO). Mitochondria were isolated from BAT essentially as described previously (17). Briefly, axillary BAT was excised from the animal and rapidly transferred to an excess of ice-cold homogenization buffer [250 mM sucrose and 5 mM *N*-tris(hydroxymethyl)-2-aminoethanesulfonic acid, pH 7.2 (K-TES)]. The tissue was then minced with a razor blade on a glass plate over ice and transferred to a Dounce tissue homogenizer. The tissue was homogenized with five to six strokes of a loose-fitting pestle, filtered through two layers of gauze, and centrifuged at 8,500 *g* for 10 min. The lipid layer was removed by aspiration, the supernatant was discarded, and the lipid remaining on the inside walls of the tube was removed using a paper tissue. The pellet was resuspended in homogenization buffer and centrifuged at 800 *g* for 10 min, and the resulting supernatant was transferred to a new tube and centrifuged at 8,500 *g* for 10 min. The crude mitochondrial pellet was then resuspended in homogenization buffer containing 0.5% fatty acid-free BSA to chelate endogenous fatty acids. This suspension was centrifuged at 8,500 *g* for 10 min, and the final mitochondrial pellet was resuspended in a buffer to expand the mitochondrial matrix (100 mM KCl and 5 mM K-TES, pH 7.2). Integrity of isolated mitochondria was estimated by duplicate measurements of the respiratory control ratio, defined as state 3 respiration (in the presence of 250 μM ADP) divided by state 4 respiration (see below). In BAT mitochondria isolated by this procedure, respiratory control ratios were always >2 in the presence of 2 mM GDP, which blocks the proton leak pathway in UCP1. Skeletal muscle mitochondria were isolated exactly as described previously (2) and always had respiratory control ratios >3.5.

Protein concentration of all mitochondrial preparations was determined in duplicate by bicinchoninic acid assay (Pierce Biotechnology) with BSA as the standard.

Proton leak assay. A convenient method to assess mitochondrial uncoupling involves parallel measurements of state 4 oxygen consumption and mitochondrial membrane potential ($\Delta\psi_m$); under these conditions, the only avenue for return of protons to the mitochondrial matrix is a specific transport protein (such as UCP1) or the idiopathic proton leak pathway endogenous to all mitochondria (11). A dual-channel chart recorder (Kipp and Zonen, Bohemia, NY) was interfaced with an oxygen electrode (model 10, Rank Brothers) to record oxygen consumption and an ion-specific electrode to measure $\Delta\psi_m$, determined 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 ([TPMP]) in the assay buffer ($[\text{TPMP}]_{\text{external}}$), a standard curve of [TPMP] and chart recorder distance was generated with incremental additions of TPMP (1, 2, 3, 4, and 5 μM) for each assay (10). Mitochondrial TPMP binding corrections were 0.2 for BAT (38) and 0.35 for skeletal muscle (42). Electrode drift was corrected after each run by addition of 2 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone. All assays were performed in triplicate.

Mitochondria (1 and 0.5 mg protein/ml for BAT and skeletal muscle, respectively) were assayed at 37°C in buffer containing 120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES, 1 mM EGTA, and 0.3% fatty-acid free BSA (pH 7.2); it was assumed that this buffer contained 406 nmol O/ml (41). All proton leak assays contained 5 μM rotenone (to inhibit oxidation of endogenous NADH), 1 μg oligomycin/ml assay buffer (to inhibit the F_0F_1 ATP synthase and, therefore, establish state 4 conditions), and 80 ng nigericin/ml assay buffer (to clamp ΔpH at 0); these additions were allowed to equilibrate with mitochondria for ≥1 min before analysis. Succinate (5 mM) was used as a substrate in all experiments, and malonate was added in 0.3 mM increments to titrate the $\Delta\psi_m$; BAT mitochondria were assayed in the absence or presence of 2 mM GDP, which was the minimum concentration of GDP required to completely inhibit proton leak (data not shown). Skeletal muscle mitochondria were assayed in the presence of 2 mM $MgCl_2$ (14). Representative traces for the proton leak assay in BAT mitochondria are shown in Fig. 1.

Statistical analysis. Data were analyzed using SAS for Windows (SAS Institute). Values are means ± SE. Levels of serum NEFA and expression levels of UCPs were analyzed using a two-way ANOVA with housing temperature (5°C or −10°C) and activity state (ACT,

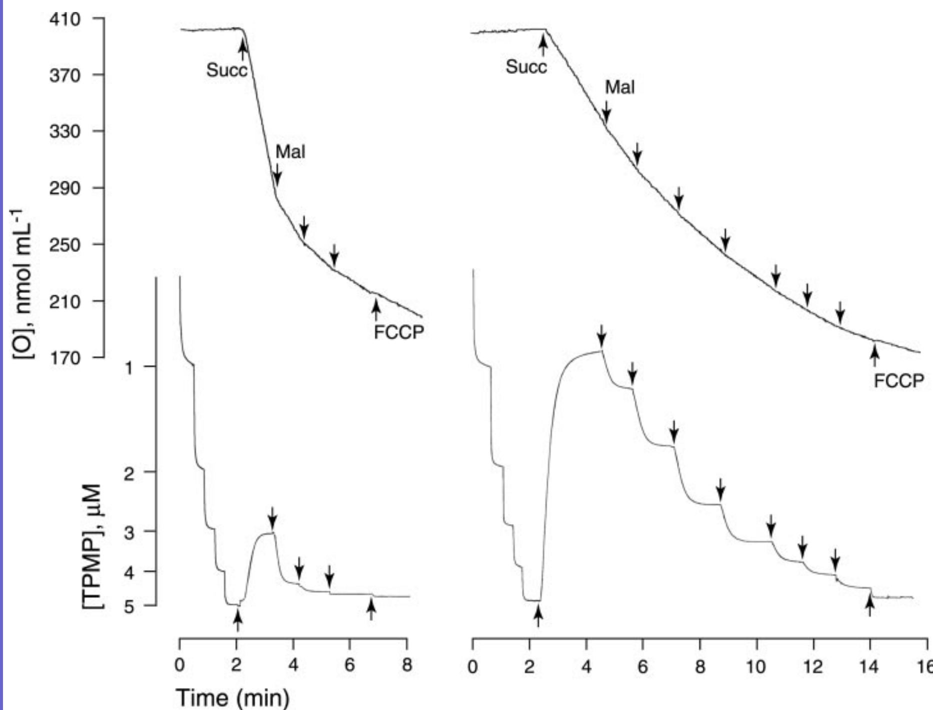

Fig. 1. Representative traces of proton leak assay in brown adipose tissue (BAT) showing relation between concentrations of oxygen and triphenylmethylphosphonium (TPMP) in assay buffer. Mitochondria from each animal were assayed in the absence (*left*) and presence (*right*) of 2 mM GDP. Mitochondria were incubated in assay buffer, rotenone, oligomycin, and nigericin for ≥1 min before addition of TPMP. Arrows designate addition of succinate (Suc), successive additions of malonate (Mal), or final addition of the respiratory uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP). Mitochondrial membrane potential ($\Delta\psi_m$) is calculated from and inversely proportional to TPMP concentration in assay buffer, such that a low concentration of TPMP in the buffer reflects a high $\Delta\psi_m$.

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Table 1. *Tissue temperatures in arctic ground squirrels hibernating under thermogenic conditions*

Tissue/Region	Temperature, °C
Brain	3.9±0.5*
BAT	3.5±0.7*
Neck	3.4±0.4*
Liver	0.2±0.5†
Rectum	0.2±0.4†
AbWAT	−0.6±0.4†‡
SkM	−1.5±0.4‡

Values are means ± SE of 7 arctic ground squirrels housed at −10°C. BAT, brown adipose tissue; abWAT, abdominal white adipose tissue; SkM, skeletal (gastrocnemius) muscle. Different symbols (*, †, ‡) indicate significant difference ($P < 0.01$).

FAST, and HIB) as main effects; post hoc comparisons among means were made using least-squares means. Tissue temperatures of hibernating arctic ground squirrels were compared by ANOVA. Comparisons between fed and fasted animals (UCP/proton leak experiments) were made using Student's *t*-tests.

RESULTS

Body and tissue temperatures. The mean T_{re} of all hibernators housed at 5°C was significantly greater than that of all hibernators housed at −10°C (5.6 ± 0.2 vs. 0.4 ± 0.3°C, $P < 0.0001$), with three animals in the latter group having a T_{re} slightly <0°C. The mean temperature of multiple tissues from seven animals housed at −10°C was significantly different among regions (Table 1). Average temperatures were higher in brain, axillary BAT, and neck than in all other regions. Average temperatures in rectum, liver, and abdominal white adipose tissue were similar to each other but lower than in brain, axillary BAT, and neck. Average gastrocnemius temperature (−1.5°C) was similar to abdominal white adipose tissue temperature but was significantly lower than temperatures of all other regions.

Patterns of Ucp1 expression and abundance of UCP1. Levels of *Ucp1* mRNA were significantly greater in animals exposed to an ambient temperature of −10°C than in animals exposed to 5°C ($P < 0.0001$). Activity state significantly affected *Ucp1* mRNA at −10°C and 5°C ($P < 0.001$), but the effect of activity state on *Ucp1* mRNA levels was statistically similar for each housing temperature (Fig. 2*A*). At the protein level, however, there was a significant interaction between the effects of housing temperature and activity state (Fig. 2*B*): hibernators housed at −10°C had the highest UCP1 levels, followed by active animals housed at −10°C and 5°C. Levels of BAT UCP1 were lowest in hibernators housed at 5°C and both of the fasted groups. *Ucp1* mRNA was not detected in skeletal muscle (data not shown).

Relation between UCP1 and proton leak. To assess the relation between fasting-induced changes in UCP1 and mitochondrial proton leak, we quantified the levels of UCP1 protein in the same tissues from which mitochondria were isolated. When measured in isolated mitochondria, fasting marginally ($P = 0.05$) decreased levels of UCP1 (Fig. 3*A*). The rate of proton leak is maximal under state 4 conditions, and in the absence of GDP the maximal leak rate and membrane potential were not significantly different between fed and fasted groups (Fig. 3*B*). Similarly, maximal proton leak rate and membrane potential were not significantly different between treatments in the presence of 2 mM GDP. However, addition of 2 mM GDP significantly decreased state 4 oxygen consumption and increased state 4 membrane potential ($P < 0.01$), although the magnitude of these changes did not differ between fed and fasted groups. At 0 and 2 mM GDP, the overall kinetics of the proton leak in BAT mitochondria were unaffected by fasting-induced decreases in UCP1, inasmuch as oxygen consumption did not differ between fed and fasted groups at common values of $\Delta\psi_m$ (Fig. 3*B*).

Patterns of Ucp3 expression. Levels of *Ucp3* mRNA in skeletal muscle were unaffected by the degree of cold expo-

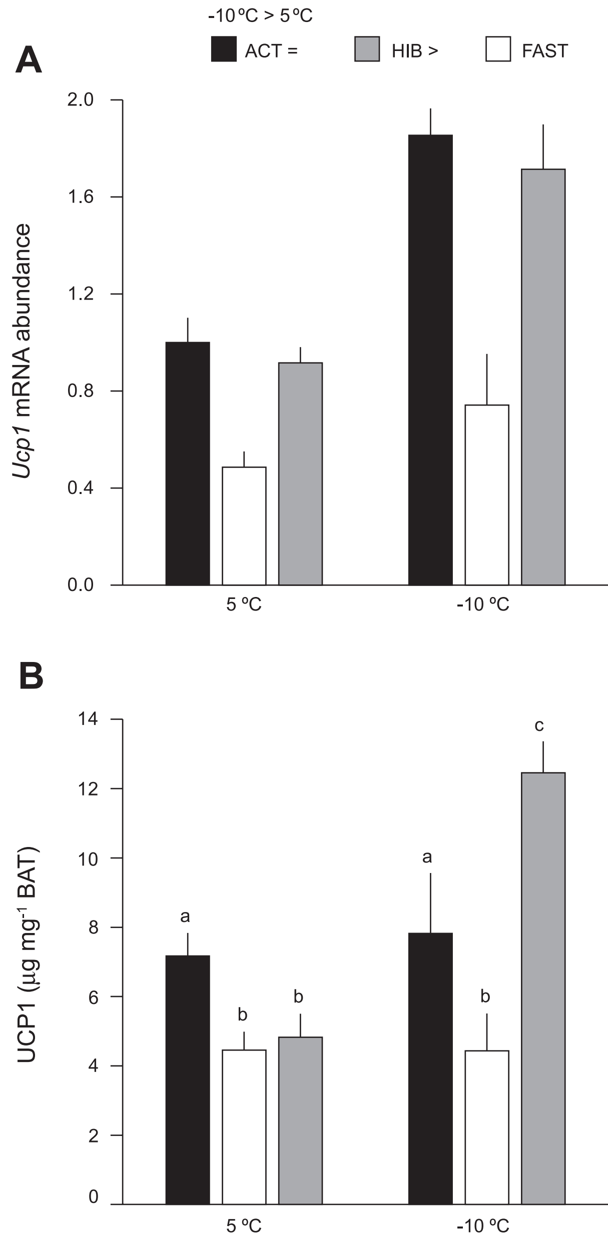

Fig. 2. Effect of temperature and activity state on levels of BAT uncoupling protein 1 (*Ucp1*) mRNA standardized to 18S rRNA (*A*) and UCP1 quantified from BAT homogenates (*B*) in arctic ground squirrels. Active animals that had not hibernated (ACT), active animals subjected to a 48-h fast (FAST), and hibernating animals in deep torpor (HIB) were housed at 5°C or −10°C. Values are means ± SE. Means with different letters (a–c) are significantly different ($P < 0.05$).

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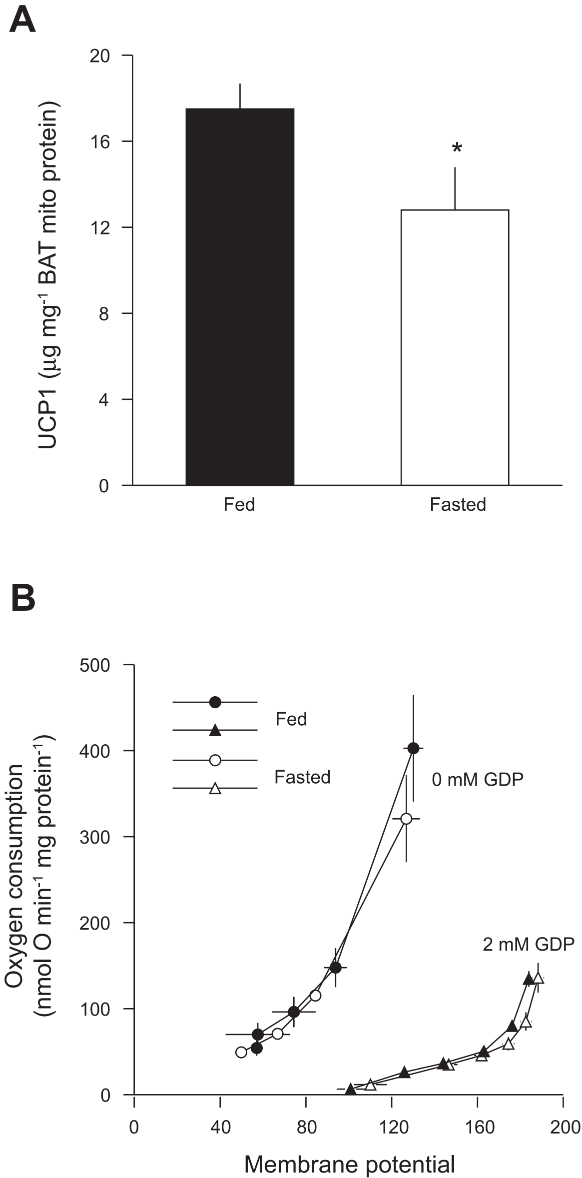

Fig. 3. Levels of UCP1 protein (*A*) and proton leak kinetics (*B*) in BAT mitochondria isolated from fed and fasted arctic ground squirrels housed at 5°C. Proton leak was measured at 0 and 2 mM GDP. Maximal rate of proton leak occurs under state 4 conditions, which are shown as extreme upper-right point for each curve; neither oxygen consumption nor membrane potential was significantly different between 0 and 2 mM GDP. There was a significant effect of GDP on state 4 oxygen consumption ($P < 0.01$) and membrane potential ($P < 0.001$) for fed and fasted groups. Values are means ± SE. *Significantly different from Fed ($P = 0.05$).

sure. However, *Ucp3* mRNA abundance was significantly different among activity states, with the highest levels in fasted animals, intermediate levels in hibernating animals, and the lowest levels in active animals ($P < 0.0001$; Fig. 4). This trend among activity states was statistically similar for each housing temperature (no significant interaction between housing temperature and activity status). *Ucp3* mRNA was not detected in BAT (data not shown).

Relation between UCP3 and proton leak. Using mitochondrial protein isolated from frozen tissue and from previously frozen mitochondria, we attempted to quantify UCP3 protein; however, UCP3 was consistently degraded and, consequently, unsuitable for quantification. Therefore, we measured levels of UCP3 protein in mitochondria freshly isolated from two separate groups of animals that were treated the same as the animals used in the proton leak experiments. A 48-h fast significantly increased levels of UCP3 protein (Fig. 5*A*). However, maximal rates of proton leak and membrane potential (state 4 conditions) were not significantly different between fed and fasted groups. Similarly, the overall kinetics of the mitochondrial proton leak were similar between groups, inasmuch as oxygen consumption was not significantly different between groups at common values of $\Delta\psi_m$ (Fig. 5*B*).

Serum NEFA. Serum NEFA levels were not significantly different between housing temperatures. NEFA levels were significantly affected by activity class ($P < 0.0001$), with the highest concentration in hibernating animals and lower concentrations, which were not significantly different from each other, in active and fasted groups. The trend among activity states was statistically similar for each housing temperature (Fig. 6).

DISCUSSION

The overall goal of this study was to determine whether UCP homologs mediate NST during hibernation by integrating measurements at the molecular, subcellular, and tissue levels. Consistent with its established role in mediating NST, levels of *Ucp1* mRNA and UCP1 in BAT were increased during cold exposure and decreased after fasting. In contrast, levels of *Ucp3* mRNA in skeletal muscle did not change as a function of cold exposure, and levels of *Ucp3* mRNA and UCP3 increased after a 48-h fast. Despite a nearly fivefold increase in UCP3, mitochondrial proton leak was unchanged. Furthermore, gastrocnemius muscle temperature was significantly lower than BAT temperature in squirrels housed at −10°C. Together, these data do not support the hypothesis that UCP3 is a

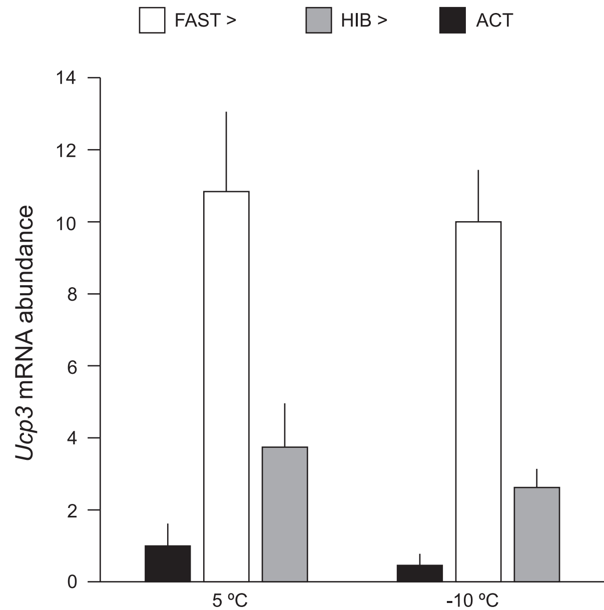

Fig. 4. Effect of temperature and activity level on skeletal muscle *Ucp3* mRNA standardized to 18S rRNA in arctic ground squirrels housed at 5°C or −10°C. Note significant effects of activity class on *Ucp3* mRNA levels ($P < 0.0001$). Values are means ± SE.

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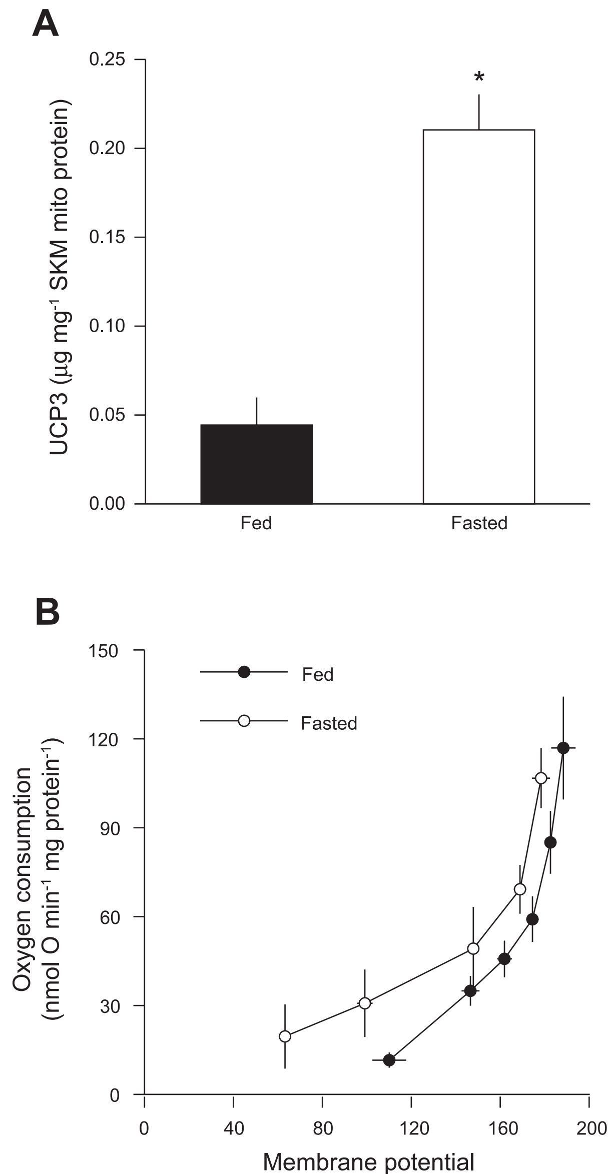

Fig. 5. Levels of UCP3 protein (*A*) and proton leak kinetics (*B*) in skeletal muscle (SKM) mitochondria isolated from fed and fasted arctic ground squirrels housed at 5°C. Values are means ± SE. *Significantly different from Fed ($P < 0.01$). Rate of proton leak is maximal under state 4 conditions, which are shown as extreme upper-right point for each curve; neither oxygen consumption nor membrane potential was significantly different between treatments.

mediator of NST in skeletal muscle; rather, the increased levels of *Ucp3* mRNA during fasting and hibernation are more readily explained by the hypothesized role of UCP3 as a mediator of fatty acid metabolism.

Regulation of Ucp1 by temperature, fasting, and hibernation. Cold exposure increases thermogenic capacity of BAT via mitochondrial biogenesis and increases in UCP1 content (32, 47); conversely, the amount and activity of UCP1 are decreased during fasting to promote metabolic efficiency (39, 48). We exploited this paradigm in the arctic ground squirrel by housing animals under one of two ambient temperatures (5°C and −10°C) and measuring thermogenic parameters in one of three activity states (hibernating, not hibernating and fasted for 48 h, and nonhibernating). The molecular data clearly support the hypothesis that UCP1 is a mediator of NST: *Ucp1* mRNA and UCP1 levels were significantly greater in cold-exposed animals (−10°C vs. 5°C). Consistent with studies in mice (39, 48) and rats (18), a 48-h fast decreased levels of *Ucp1* mRNA and UCP1 in arctic ground squirrels (Fig. 2). However, the magnitude of the fasting-induced decrease in *Ucp1* mRNA levels was not different between housing temperatures.

During steady-state hibernation at >0°C, thermogenesis is minimal, body temperature parallels ambient temperature, and the role of UCP1 is confined to rewarming to a high body temperature during periodic arousal bouts by activating the preexisting UCP1 (37). We observed a similar absence of NST in hibernators housed at 5°C: the average T_{re} of these animals was 5.6 ± 0.2°C, and levels of *Ucp1* mRNA and UCP1 did not differ between the ACT and HIB groups (Fig. 2). In contrast to the pattern at 5°C, UCP1 protein was significantly increased in hibernators housed at −10°C compared with all other groups. Measurements of BAT temperature highlight the importance of UCP1-mediated NST during thermogenic hibernation. The average temperature of the axillary BAT pad of hibernators exposed to −10°C was 3.5 ± 0.7°C, which is ∼14°C warmer than the ambient temperature and ∼5°C warmer than the coldest tissue measured, i.e., gastrocnemius muscle (Table 1). Interestingly, levels of UCP1 were lower in ACT and FAST than in HIB squirrels maintained at −10°C, despite the larger thermal gradient between their high body temperature and the environment (Fig. 2*B*). Increased shivering thermogenesis, incompatible with hibernation, may compensate for the lack of UCP1-mediated NST in these animals.

UCP1-mediated proton leak. In a separate group of animals used for measuring mitochondrial proton leak, UCP1 protein was measured in isolated mitochondria (as opposed to whole

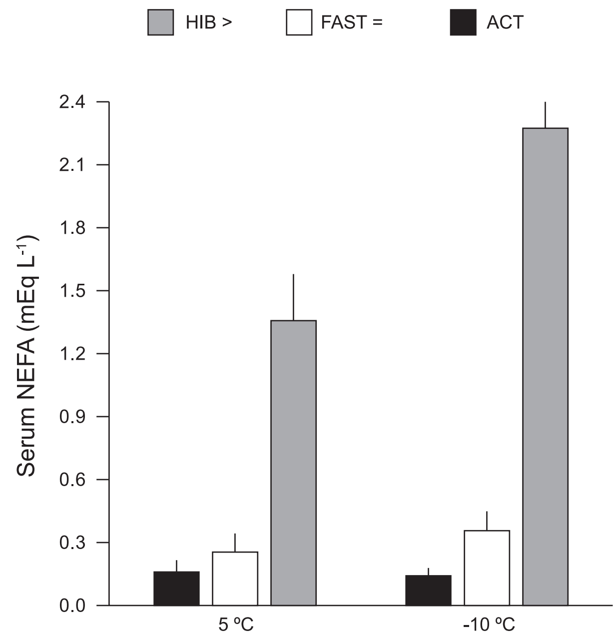

Fig. 6. Effect of temperature and activity state on serum levels of nonesterified fatty acids (NEFA) in arctic ground squirrels housed at 5°C or −10°C. NEFA levels were significantly different among all activity states ($P < 0.0001$). Values are means ± SE.

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tissue homogenates; see above), and we observed a 27% reduction in UCP1 content, which was marginally different ($P = 0.05$; Fig. 3*A*). Despite the reduction in the amount of UCP1, the kinetics of the proton leak assayed in BAT mitochondria in the absence of GDP were identical in fed and fasted groups: mitochondrial membrane potential was not increased, and respiration was not decreased (Fig. 3*B*). It is unlikely that the inability to resolve a difference in proton leak at 0 mM GDP was due to disruption of the mitochondrial membrane during isolation, inasmuch as proton leak in the same mitochondria was markedly reduced in the presence of 2 mM GDP: oxygen consumption at a membrane potential of ~130 mV was ~10-fold lower in the presence of 2 mM GDP (Fig. 3*B*). The inability to detect changes in proton leak at 0 mM GDP could be due to the persistence of UCP1 protein in the fasted group (~12 μg UCP1/mg mitochondrial protein). Although the level of UCP1 protein was reduced by 27% compared with the fed group, the amount of UCP1 in the fasted group may have been sufficient to allow mitochondrial uncoupling that was indistinguishable from that of the fed group when measured in vitro. We are not aware of any study describing the relation between mitochondrial proton leak in BAT and physiological changes in UCP1; therefore, it is possible that this method of mitochondrial proton leak estimation is of limited utility for study of tissues with a large capacity for mitochondrial uncoupling in vivo. Nonetheless, further studies are needed to determine the relation between levels of UCP1 protein and mitochondrial proton leak in BAT.

Regulation of Ucp3 by temperature, fasting, and hibernation. We observed that the regulation of *Ucp3* was inconsistent with a role in NST, wherein *Ucp3* mRNA levels were not increased with cold exposure and were increased ~10-fold after a 48-h fast (Fig. 4). Interestingly, levels of *Ucp3* mRNA were significantly increased more than threefold in hibernators compared with fed nonhibernators; this is similar to a previous study in this species, where it was proposed that UCP3 may mediate NST in skeletal muscle during hibernation (8). However, we show in this study that an increase in *Ucp3* expression is inconsistent with a role in NST during hibernation: *Ucp3* mRNA levels were substantially higher in fasted animals when energy metabolism should be reduced, and the pattern of *Ucp3* expression was similar between animals housed at warm and cold temperatures. Furthermore, the temperature of skeletal muscle in hibernators housed at −10°C was significantly lower than the temperature of BAT, a known site of NST; in fact, the temperature of skeletal was the lowest of all tissues measured, averaging −1.5°C (Table 1). The increased temperature of skeletal muscle relative to the ambient temperature is more likely achieved by perfusion of blood warmed by BAT (23), rather than a local increase in mitochondrial uncoupling.

UCP3-mediated proton leak. In the proton leak experiments, the level of UCP3 protein in isolated mitochondria was elevated nearly fivefold after a 48-h fast (Fig. 5*A*). Despite the marked increase in UCP3 protein, proton leak kinetics in mitochondria isolated from skeletal muscle were unchanged (Fig. 5*B*). These data agree with other studies where changes in proton leak in skeletal muscle mitochondria did not correlate with diet-induced changes in UCP3 (5, 15, 31) but are inconsistent with other studies that suggest that mitochondrial proton leak is increased in yeast expressing human *Ucp3* (30, 54) and transgenic mice overexpressing human *Ucp3* (19). However, it has now been clearly demonstrated that increased proton leak observed with UCP3 overexpression is due to artifactual uncoupling: increased proton leak is only observed in yeast when UCP3 levels are sevenfold higher than in wild-type mice, whereas proton leak is not changed when yeast express UCP3 at levels seen in fasted mice (28). Moreover, in transgenic mice showing increased proton leak, UCP3 levels are 20-fold higher than in wild-type mice (16). Importantly, recent studies of mice with UCP3 levels elevated within the physiological range demonstrate no change in proton leak (35), and an increase in UCP3 induced by long-term calorie restriction is associated with decreased rate of mitochondrial proton leak (5). Because it has been well demonstrated that proton leak does not change with UCP3 protein levels in the physiological range, the unchanged proton leak observed in this study is likely to be real and not due to any methodological considerations (as opposed to the measurement of proton leak in BAT; see above). Furthermore, we observed a lower expression of *Ucp3* in hibernating than in fasted animals (Fig. 4), providing additional evidence that UCP3 is not a physiologically relevant mediator of NST during hibernation.

Role of Ucp3 in fatty acid metabolism. The hypothesis that UCP3 plays a role in fatty acid handling (29) has garnered the most experimental support to date. Expression of *Ucp3* is stimulated by fasting-induced increases in fatty acids (49) and by lipid infusion (52), and mice overexpressing UCP3 within the physiological range (~2.5-fold) show enhanced capacity for fatty acid oxidation (6). In arctic ground squirrels, fasting induced a 10-fold increase in skeletal muscle *Ucp3* mRNA (pooled across temperature groups) and a 5-fold increase in UCP3 protein, although the resulting 2-fold increase in NEFA levels was not significantly different from that in active squirrels (Fig. 6). It is possible that the elevated levels of *Ucp3* mRNA and UCP3 protein effectively increased fatty acid oxidation to the extent that NEFA levels were similar between fed and fasted animals; Bezaire et al. (6) reported that serum NEFA levels are significantly lower in transgenic mice exhibiting a 2.5-fold increase in UCP3 than in controls. We observed the highest levels of NEFAs in hibernating squirrels (>5-fold increase compared with fasted animals); yet skeletal muscle *Ucp3* mRNA levels in hibernators were one-third of the levels in fasted squirrels. It is unclear why the high levels of NEFA in hibernators does not translate to increased *Ucp3* expression relative to fasted animals, but a likely explanation is that skeletal muscle is metabolically quiescent during hibernation and, thus, the expression of metabolic genes is unwarranted. The observation that skeletal muscle temperature is −1.4°C in hibernators housed at −10°C supports the hypothesis that substrate oxidation is minimal in skeletal muscle during hibernation. Furthermore, this extremely low temperature casts doubt on any potential NST mediated by UCP3 in this tissue.

In summary, we have shown that expression of *Ucp3* in skeletal muscle sharply contrasts with expression of *Ucp1* in BAT and is inconsistent with a role in thermogenesis: *Ucp3* expression is unaffected by cold exposure and is increased with fasting, whereas *Ucp1* expression and UCP1 protein levels are increased with cold exposure and decreased with fasting. Furthermore, temperature measurements of these tissues suggest that BAT is the primary source of thermogenesis during hibernation, whereas the skeletal muscle temperature is extremely low, despite an increase in the level of *Ucp3* mRNA.

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It is possible that, using alternative assay conditions, we may have observed an increase in UCP homolog-mediated proton leak; however, any uncoupling of oxidative phosphorylation through UCP3 is unlikely to be thermogenic because of the very low absolute abundance of UCP3 compared with UCP1 (>300-fold lower in fed animals; cf. Figs. 3*A* 5*A*). Although our findings refer specifically to the role of UCP homologs during hibernation, they are consistent with the growing body of evidence suggesting that these proteins are not likely to be physiologically relevant mediators of NST.

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