Differential regulation of uncoupling protein gene homologues in multiple tissues of hibernating ground squirrels

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Boyer, Bert B., Brian M. Barnes, Bradford B. Lowell, and Danica Grujic. Differential regulation of uncoupling protein gene homologues in multiple tissues of hibernating ground squirrels. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1232–R1238, 1998.—Nonshivering thermogenesis in brown adipose tissue (BAT) provides heat through activation of a mitochondrial uncoupling protein (UCP1), which causes futile electron transport cycles without the production of ATP. Recent discovery of two molecular homologues, UCP2, expressed in multiple tissues, and UCP3, expressed in muscle, has resulted in investigation of their roles in thermoregulatory physiology and energy balance. To determine the expression pattern of Ucp homologues in hibernating mammals, we compared relative mRNA levels of Ucp1, -2, and -3 in BAT, white adipose tissue (WAT), and skeletal muscle of arctic ground squirrels (Spermophilus parryii) hibernating at different ambient and body temperatures, with levels determined in tissues from ground squirrels not in hibernation. Here we report significant increases in mRNA levels for Ucp2 in WAT (1.6-fold) and Ucp3 in skeletal muscle (3-fold) during hibernation. These results indicate the potential for a role of UCP2 and UCP3 in thermal homeostasis during hibernation and indicate that parallel mechanisms and multiple tissues could be important for nonshivering thermoregulation in mammals.

nonshivering thermogenesis; Spermophilus; uncoupling protein 1; uncoupling protein 2; uncoupling protein 3

UNTIL RECENTLY, nonshivering thermogenesis in placental mammalian neonates and hibernators has been thought to originate principally through activation of a mitochondrial uncoupling protein (UCP1), specific to brown adipose tissue (BAT) (27). Heat production by BAT can play a major role in thermoregulation and energy balance. When rodents and other small mammals are exposed to cold, the amount and activity of UCP1 increases in BAT (24, 25). Ucp1 knockout mice are cold sensitive, indicating that their thermoregulation is defective (10), and expression of Ucp1 in white adipose tissue (WAT) results in the prevention of genetic obesity in mice (18). In addition, increases in BAT temperature and conductance of protons during warming from torpor demonstrate the involvement of BAT during nonshivering thermogenesis in hibernators (22). Recently, two molecular homologues to UCP1, called UCP2 and UCP3, have been identified in human and rodent tissues (6, 11, 14, 32), opening the possibility that nonshivering thermogenesis and energy balance may be affected by more than one mitochondrial uncoupler and in multiple tissues.

UCP2 is expressed in several different tissues in mice, rats, and humans, and it functions as a mitochondrial uncoupler when expressed in yeast (11, 14, 15). Ucp2 mRNA levels in WAT are higher in genetic models of obesity (14), and they increase in response to high-fat diet (11) and after administration of leptin (33). Cold exposure increases Ucp2 mRNA levels in heart, BAT, and muscle of rats (4), but not in BAT, WAT, muscle or liver of mice (11).

UCP3 is expressed at high levels in human skeletal muscle and in rat and mouse BAT and skeletal muscle (6, 32), and it also functions as a mitochondrial uncoupler when expressed in yeast (15) or C2C12 myoblasts (5). Ucp3 mRNA levels in muscle are not increased by cold exposure (6), but they do significantly increase in WAT following treatment with the β3-adrenergic agonist CL214613 (15). In addition, muscle Ucp3 mRNA levels are increased during 48 h of fasting, and by administration of thyroid hormones, glucocorticoids, and leptin in rats (15). Although UCP1, -2, and -3 protein concentrations were not measured in these studies, the broad tissue distribution pattern of Ucp2 mRNA and strong expression of Ucp3 mRNA in muscle suggest that these proton translocators may participate in overall thermogenesis and energy balance in mammals (6, 11, 14, 32).

The existence of multiple candidate thermogenic effectors suggests that more than one UCP homologue and tissue may contribute to nonshivering heat production, particularly in hibernating mammals. Hibernation is the period of seasonal heterothermy that consists of torpor (low body temperature) and recurring arousal episodes, each of which includes three phases: rewarming from torpor, euthermia, and recooling into torpor. During hibernation in ground squirrels, body temperature (Tb) falls to <10% of normal levels (2). This reduction does not represent defective thermoregulation. Under decreasing ambient temperatures, metabolic rate and heat production rise in hibernating mammals in defense of a lowered Tb set point (16) and keep torpid animals from freezing as ambient temperatures decrease below 0°C (13). Regulated thermogenesis is also necessary to fuel arousal episodes. These significant thermogenic demands represent challenges necessary for overwinter survival, especially in environments where hibernaculum temperatures are regularly and significantly below freezing (1).

The aim of the present study was to determine the expression pattern of Ucp homologues and whether...
Ucp2 and Ucp3 mRNA levels are differentially regulated in arctic ground squirrels (Spermophilus parryii) maintained at different ambient temperatures and in different states of hibernation.

MATERIALS AND METHODS

Animals. Arctic ground squirrels were trapped in the Alaska Range (64°N, elevation 900–1,200 m) or on the North Slope of Alaska near Toolik Lake (68°38′N, 149°38′W, elevation 809 m), transported to University of Alaska Fairbanks, and housed at 18°C on an 18:6-h light-dark cycle. In the fall animals were housed in an environmental chamber at 4 ± 1°C and on an 8:16-h light-dark cycle in cages with cotton nest batting. Mazuri Rodent Chow, sunflower seeds, and water were provided ad libitum. Blood samples were obtained from ground squirrels following anesthesia with methoxyflurane, and tissue samples were collected after death by an intracardiac injection of pentobarbital sodium. All tissues were frozen in liquid nitrogen within 5 min of death and stored at −70°C until total RNA was isolated at a later date. Animal protocols were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee.

Telemetry and data logging of body temperature. In some of the animals, activity/temperature-sensitive radiotransmitters (model VMF-BB; Mini-Mitter, Sunriver, OR) were used to record Tb (2). At death, Tb was verified with a rectal thermometer. “Hibernating” ground squirrels were either torpid (Tb, −1 to 7°C for 1–15 days) or in the euthermic phase of an arousal episode (Tb, 32 to 37°C for 1–15 h). Ground squirrels “not hibernating” were either maintained at 20°C or cold exposed to 0°C for 5 days and were killed in the lean phase of their annual cycle (March and June, respectively) (23). An additional record of Tb of a free-living adult male arctic ground squirrel overwintering near Toolik Lake was collected from September to January by a miniature data logger (modified Tidbit logger; Onset, Pocasset, MA) implanted in its abdominal cavity on August 30. The animal was held in captivity for 48 h postoperation before being released at its own burrow. This animal was recaptured the following spring, the logger was surgically removed and downloaded, and the animal was released again. In addition, soil temperature was recorded each 4.8 h for soil at −0.9 m (permafrost table depth) near this animal’s burrow with HoboTemp loggers (Onset).

Northern analysis. Total RNA was isolated and analyzed as described (7) with minor modifications. Tissues obtained for Northern analysis included interaxillary BAT, intra-abdominal WAT, muscle (gastrocnemius), liver, kidney, spleen, heart, and brain. An oligonucleotide probe was used to detect Ucp1 mRNA (8). The Ucp2 and Ucp3 cDNA probes have been previously described (32). After transfer to Hybond N+ membranes (Amersham), blots were hybridized for 12 h at 45°C in a solution containing 50% formamide, 5× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH2PO4, and 1 mM EDTA), 5× Denhardt’s solution [1× Denhardt’s solution is 1% Ficoll, 1% bovine serum albumin (fraction V), and 1% polyvinylpyrrolidone], 1% SDS, 100 µg/ml salmon sperm DNA, and −1 × 106 cpm/ml labeled probe. Blots were washed two times at room temperature in 2× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.2% SDS. Higher-stringency washes were with 0.1× SSC, 0.2% SDS one time at 37°C or additional times at higher temperatures until the background radioactivity was reduced. Blots were exposed with intensifying screens to Hyperfilm MP film (Amersham) at −70°C for 3–8 days. All blots were reprobed with an 18S rRNA restriction fragment to allow us to normalize for variations in RNA loading. Relative changes in Ucp1, -2, and -3 mRNA levels were determined by densitometry scanning of autoradiographs. All autoradiograph exposures used for densitometry were in the linear range of the film exposure. To compare densitometry representing Ucp2 mRNA levels, we reexposed the film for a shorter period of time and compared the hibernating group to the nonhibernating group (animals exposed to 20°C only). This was necessary because Ucp2 mRNA levels in WAT of animals housed at 0°C were too low to detect by densitometry when the hibernating group’s mRNA levels were in the linear range of the film.

Cross-hybridization between Ucp gene homologues is possible and has been shown between Ucp2 and Ucp3 (6, 32). However, Ucp2 and Ucp3 have significantly different transcript sizes (Fig. 3). No cross-hybridization has been observed between Ucp2 or Ucp3 and Ucp1 when full-length cDNA probes were used. Finally, although we used a 27-bp synthetic oligonucleotide probe to detect the Ucp1 transcript, the Ucp1 gene has three polyadenylation signal sequences, resulting in two visible transcripts characteristic of this homologue (for more details see Ref. 20). Therefore, the mRNA sizes and banding characteristics for Ucp1, Ucp2, and Ucp3 are sufficiently different to differentiate between these homologues by Northern analysis.

Statistics. The relative levels of Ucp1, -2, and -3 mRNA in hibernating animals (torpid and the euthermic phase of an arousal episode) were compared with those in animals not hibernating (exposed to 0 or 20°C) by a one-tailed Student’s t-test. Differences were considered significant at P < 0.05. The data are expressed as means ± SE.

RESULTS

To indicate the range of thermoregulatory states arctic hibernators face during winter, a record of Tb changes for a representative arctic ground squirrel is shown for the first half of its hibernation season (Fig. 1). Minimum Tb during torpor decreased in autumn in parallel with the fall in soil temperature,
indicating passive thermal equilibrium between the animal and its surroundings when ambient temperatures were above freezing. In early December, when soil temperatures decreased from approximately −4°C to −10°C, the ground squirrel’s T_b remained constant during torpor at a minimum of −2.0°C, indicating active thermoregulation. Throughout hibernation, bouts of torpor were regularly interrupted by periodic arousal episodes every 10–21 days, each requiring substantial thermogenesis and active thermoregulation.

The tissue distribution of Ucp1, -2, and -3 mRNAs in euthermic ground squirrels housed at 20°C and not in hibernation is shown in Fig. 2. Ucp1 was expressed exclusively in BAT. Highest levels of Ucp2 mRNA among tissues compared were detected in WAT and spleen, with lower levels detectable in BAT, heart, and kidney. Ucp3 mRNA was detected at low levels and only in skeletal muscle of animals housed at 20°C.

To determine whether the Ucp homologues were differentially expressed during hibernation, we compared relative mRNA levels of Ucp1, -2, and -3 in BAT, WAT, and skeletal muscle of arctic ground squirrels hibernating at different ambient and body temperatures to levels found in tissues from ground squirrels in the lean phase of their annual body weight rhythm and not in hibernation. Ucp1 mRNA levels in BAT of hibernating squirrels (torpid and during the euthermic phase of an arousal episode) were similar to levels in squirrels not hibernating (exposed to 20 and 0°C) (Figs. 3 and 4). In contrast, Ucp2 mRNA levels in WAT were 1.6-fold greater in hibernating squirrels compared with squirrels not in hibernation (exposed to 20°C). Although it appears from Fig. 3 that the induction of Ucp2 mRNA in hibernating animals was greater than a 1.6-fold increase, it should be noted that the Northern blot only includes animals for which we had all three tissues, whereas the bar graphs (Fig. 4) represent means and SE based on the analysis of additional individuals (number is noted under each bar). Low but detectable Ucp3 mRNA levels in skeletal muscle of ground squirrels that were not hibernating increased threefold in hibernating ground squirrels (Figs. 2–4).

To investigate whether Ucp1, -2, and -3 mRNAs may be increased in additional tissues in hibernating squirrels, we reprobed the skeletal muscle, BAT, and WAT blots (shown in Fig. 3) with the Ucp1, Ucp2, and Ucp3 probes. We were unable to detect Ucp3 mRNA in BAT or WAT, Ucp1 in skeletal muscle or WAT, or Ucp2 mRNA in skeletal muscle of hibernating squirrels (data not shown). However, we were able to detect Ucp2 mRNA in BAT of both cold-exposed and hibernating arctic ground squirrels, although Ucp2 mRNA levels were not elevated in hibernating squirrels compared with those from squirrels that were not hibernating (Fig. 5).

Ground squirrels not hibernating and exposed to 0°C for 5 days had 3.8-fold lower Ucp2 mRNA levels in WAT (P < 0.05) compared with levels in ground squirrels not hibernating and at thermoneutrality (20°C). There were no significant differences between these same groups of squirrels in Ucp1 mRNA levels in BAT, Ucp3 mRNA levels in skeletal muscle, or Ucp2 mRNA levels in BAT. Ground squirrels hibernating (either torpid or in the euthermic phase of an arousal episode) at ambient temperatures <0°C had 2.2-fold higher Ucp1 mRNA levels in BAT (P < 0.05), 2-fold higher Ucp2 mRNA levels in BAT (P < 0.05), 2.5-fold higher Ucp2 mRNA levels in WAT (P < 0.01), and 2.5-fold higher Ucp3 mRNA levels in skeletal muscle (P < 0.001) compared with ground squirrels hibernating at ambient temperatures above 0°C (Figs. 3 and 5).

**DISCUSSION**

This study demonstrates that mRNA for two recently discovered Ucp homologues are increased in several tissues during hibernation in mammals. These are two of only eight mRNAs that have so far been shown to be differentially regulated during hibernation (3, 28–30). Our data also demonstrate that the mRNA for Ucp1, -2, and -3 are significantly elevated in actively thermoregulating ground squirrels hibernating at ambient temperatures <0°C compared with nonthermogenic animals hibernating at ambient temperatures >0°C. Although we have only measured relative changes in mRNA levels, previous studies investigating the induction of Ucp1 mRNA following cold exposure of ground squirrels demonstrated that increased mRNA concentration is paralleled by increases in UCP1 protein concentration and UCP activity (24). Therefore, the differential regulation of Ucp gene homologues under differing thermogenic conditions in hibernating arctic ground squirrels suggests a potential role for nonshivering thermogenesis and energy regulation in more than just BAT of hibernating mammals.
Voluntary aphagia is a natural behavior of hibernating arctic ground squirrels (23), and fasting has also been shown to increase Ucp3 mRNA levels in muscle but not Ucp2 mRNA levels in WAT of rats (15). It is therefore possible that the elevated Ucp3 mRNA levels in skeletal muscle we measured in hibernating arctic ground squirrels resulted from fasting associated with hibernation. However, fasting alone is not sufficient to explain the significant increases in Ucp3 mRNA levels in skeletal muscle observed in squirrels hibernating at ambient temperatures ≤0°C compared with squirrels hibernating at ambient temperatures >0°C, because both groups had been aphagic for >1 mo before tissues were collected (Fig. 3).

In mammals Ucp2 mRNA has been detected in several different tissues whereas Ucp3 mRNA is primarily expressed in skeletal muscle and BAT (6, 11, 14, 15, 32). Consistent with its expression pattern in other rodents, we observed the highest levels of Ucp2 mRNA in WAT and spleen and lower levels in heart and BAT of arctic ground squirrels (Fig. 2). However, unlike in mice or rats, we were unable to detect Ucp2 mRNA in skeletal muscle, liver or brain of arctic ground squirrels. Ucp3 mRNA was only detected in skeletal muscle of ground squirrels, and its mRNA levels were elevated threefold during hibernation (Figs. 2–4). The differences in tissue-specific expression patterns for Ucp2 and Ucp3 mRNA may reflect important differences in mitochondrial metabolism between ground squirrels and other rodents and humans.

Elevated Ucp2 and Ucp3 mRNA levels in hibernating ground squirrels could be due to increased synthesis or decreased degradation of mRNA, and these changes could occur at a number of times in the hibernation cycle. For example, mRNA synthesis may increase as part of preparation for hibernation or occur during an

Fig. 3. Northern blot of total RNA isolated from BAT (20 µg/lane), WAT (15 µg/lane), or skeletal muscle (SKM, 20 µg/lane) from arctic ground squirrels exposed to different ambient temperatures (Ta) and with different body temperatures (Tb). Autoradiographic data are presented only from animals where all 3 tissues were available.
Ucp1 mRNA was expressed exclusively in BAT (Fig. 2) and did not increase in hibernating arctic ground squirrels (torpid and interbout euthemic arousal interval) compared with squirrels not hibernating (Figs. 3 and 4). These results support earlier studies that indicate the increase in thermogenic capacity of BAT during hibernation or fall prehibernation fattening is modulated by increases in BAT mass and mitochondrial content, not UCP1 concentration (22). However, when we compared Ucp1 mRNA levels in hibernating squirrels at ambient temperatures <0°C to levels in hibernating squirrels at ambient temperatures >0°C, we found that Ucp1 mRNA levels were significantly increased. Thus, in addition to the "unmasking" of UCP1 activity during an arousal episode (24) and the constant UCP1 protein and mRNA levels in BAT of hibernating squirrels at ambient temperatures >0°C (22), our results suggest that BAT of arctic ground squirrels can respond to increased thermogenic demands (ambient temperatures <0°C) by increasing Ucp1 mRNA levels and possibly increasing UCP1 protein concentration and activity (22).

Ucp2 mRNA levels in WAT and Ucp3 mRNA levels in skeletal muscle were significantly increased in hibernating arctic ground squirrels compared with levels in ground squirrels not hibernating (Figs. 3 and 4); among hibernating animals levels were higher in ambient temperature conditions <0°C compared with ambient temperatures >0°C (Fig. 3). The potential thermoregulatory significance of an increase in WAT Ucp2 mRNA and its translation into active protein warrants discussion, because WAT does not contain the typical concentration of mitochondria found in BAT or skeletal muscle. There is evidence for WAT mitochondriogenesis following cold exposure in mice (21). WAT capillary density increases following cold exposure and the unilocular morphology of WAT changes to a multilocular appearance more typical of BAT (21). Cold exposure of mice and rats increases the expression of Ucp1 in brown adipocytes that have infiltrated BAT or skeletal muscle (9, 21). Although we do not know yet whether mitochondriogenesis or brown adipocyte conversion occurs in hibernating ground squirrels following cold stress, the appearance of even a minimal increase in UCP2 activity in white adipocytes could have a significant effect on heat production abilities of a hibernating ground squirrel. This point is illustrated in a transgenic mouse line in which Ucp1 expression was driven by the fat-specific aP2 promoter (18). While the aP2-Ucp transgenic mice showed both Ucp1 mRNA and immunoreactive UCP in BAT at only 2—10% of the levels normally found in BAT, the transgene was able to prevent both genetic obesity (18) and dietary obesity in mice (19). These data strongly suggest that WAT in the aP2-Ucp transgenic mouse was thermogenically active. Because the percent WAT in these mice represented ~14% of their total body weight (18), but 40% of the total body weight in hibernating arctic ground squirrels (12), a 1.6-fold increase in Ucp2 mRNA (if it is translated into protein and active) could significantly increase the thermogenic output of WAT in hibernating ground squirrels.

Skeletal muscle does contain significant numbers of mitochondria, and protein is the second most abundant

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**Fig. 4.** Ucp1, -2, and -3 mRNA levels were calculated from Northern blot shown in Fig. 3 and represent ratio of Ucp1, -2, or -3 photodensitometry units divided by 18S rRNA photodensitometry units. Ucp mRNA ratios were normalized to 1.0 for squirrels not hibernating (Not Hib; consists of animals exposed to 20 and 0°C). A: Bat. B: WAT. C: SKM. For comparison of Ucp2 mRNA ratios in WAT, Not Hib group consists only of animals exposed to 20°C (see MATERIALS AND METHODS). Hibernating squirrels (Hib) consist of both torpid and euthermic animals in an arousal episode. Number of animals in each group is indicated in parentheses under each bar. Normalized Ucp mRNA ratios are expressed as mean ± SE. *P < 0.05 between groups (Student's t-test).
component of body composition in ground squirrels (excluding water), comprising ~12–17% of their body weight (12). Because skeletal muscle is the most important contributor to standard metabolic rate (26) and metabolic rate increases >10-fold in torpid arctic ground squirrels housed at ambient temperatures between 0 and −16°C (C. L. Buck and B. M. Barnes, unpublished observations), we reasoned that Ucp3 may be induced in arctic ground squirrels living in environments where the soil temperature drops significantly below 0°C and where thermogenesis would become necessary to prevent freezing (Fig. 1). Our data indicate that Ucp3 mRNA levels increase threefold in skeletal muscle during hibernation (Figs. 3 and 4), and we observed the highest levels of Ucp3 mRNA in animals that were required to be continuously thermogenic while housed at −5 or −12°C (Fig. 3). With some exceptions, several of the same animals showing high skeletal muscle Ucp3 mRNA levels also showed the highest levels of Ucp1 levels in BAT and Ucp2 mRNA levels in WAT and BAT (Figs. 3 and 5). Although these data are consistent with the possibility that heat production may be produced by parallel activation of multiple UCPs in several different tissues to prevent freezing, it is uncertain whether the increase in Ucp2 and Ucp3 mRNA levels actually results in increased heat production in WAT and skeletal muscle, respectively. Both UCP2 and UCP3 function as uncouplers when expressed in yeast (11, 15); however, it has recently been shown that 3.5-fold increases in Ucp3 mRNA levels in soleus muscle of rats fasted for 48 h had no effect on soleus muscle basal heat production rate measured by in vitro microcalorimetry (5). Future studies aimed at determining heat production by skeletal muscle and WAT in continuously thermogenic hibernating ground squirrels are necessary to resolve this issue.

Perspectives

We have identified two novel gene products, Ucp2 and Ucp3, that are present at elevated levels in hibernating ground squirrels, supporting a role for differential gene expression during hibernation (29). Although our data are consistent with the possibility that multiple forms of UCP in multiple tissues could act together to regulate body temperature and energy balance in hibernating mammals, we do not yet know how and when Ucp2 and Ucp3 mRNA levels increase relative to the timing of hibernation, whether UCP2 and UCP3 protein concentration and activity also increase, or what the relative contributions of these, together with UCP1, are for total thermogenesis in hibernating mammals.

Candidate mechanisms regulating Ucp2 and Ucp3 include activation by the sympathetic nervous system and thyroid hormones (17). Cold exposure or norepinephrine-induced thermogenesis increases heat production by BAT and skeletal muscle, each tissue accounting for 50% or more of the total increase in heat production, depending on the organism and method that heat production was measured (reviewed in Ref. 17). The increase in BAT heat production has been attributed to an increase in UCP1 activity and synthesis; however, it appears that Ucp2 mRNA also increases in BAT, heart, and skeletal muscle in response to the cold (4). Rats treated with β3 agonists also increase Ucp3 mRNA in WAT (15). Recently, thyroid hormone has been shown to increase Ucp3 mRNA in skeletal muscle (15). The effects of thyroid hormone thermogenesis have been well characterized (17) and may be important for hibernators because free plasma thyroid hormone levels increase by several multiples during arousal episodes (31). Future studies are being directed at identifying changes in UCP1–3 protein levels and activity and their contribution to overall thermogenesis in BAT, WAT, and skeletal muscle in hibernating ground squirrels.

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