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 thermogenesis 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 thermogenesis 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 rewarming 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 recoring 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 thermocouple and 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 killed in the lean phase of their annual cycle (March and June, respectively) (23) (Fig. 1). Minimum Tb during torpor decreased in autumn in parallel with the fall in soil temperature, that interrupt torpor.

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 $Ucp_1$, -2, and -3 mRNAs in euthermic ground squirrels housed at 20°C and not in hibernation is shown in Fig. 2. $Ucp_1$ was expressed exclusively in BAT. Highest levels of $Ucp_2$ mRNA among tissues compared were detected in WAT and spleen, with lower levels detectable in BAT, heart, and kidney. $Ucp_3$ 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 $Ucp_1$, -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. $Ucp_1$ 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, $Ucp_2$ 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 $Ucp_2$ 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 $Ucp_3$ mRNA levels in skeletal muscle of ground squirrels that were not hibernating increased threefold in hibernating ground squirrels (Figs. 2–4).

To investigate whether $Ucp_1$, -2, and -3 mRNAs may be increased in additional tissues in hibernating squirrels, we probed the skeletal muscle, BAT, and WAT blots (shown in Fig. 3) with the $Ucp_1$, $Ucp_2$, and $Ucp_3$ probes. We were unable to detect $Ucp_3$ mRNA in BAT or WAT, $Ucp_1$ in skeletal muscle or WAT, or $Ucp_2$ mRNA in skeletal muscle of hibernating squirrels (data not shown). However, we were able to detect $Ucp_2$ mRNA in BAT of both cold-exposed and hibernating arctic ground squirrels, although $Ucp_2$ 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 $Ucp_2$ 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 $Ucp_1$ mRNA levels in BAT, $Ucp_3$ mRNA levels in skeletal muscle, or $Ucp_2$ 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 $Ucp_1$ mRNA levels in BAT ($P < 0.05$), 2-fold higher $Ucp_2$ mRNA levels in BAT ($P < 0.05$), 2.5-fold higher $Ucp_2$ mRNA levels in WAT ($P < 0.01$), and 2.5-fold higher $Ucp_3$ 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 $Ucp_1$, -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 $Ucp_1$ 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 $<0^\circ$C compared with squirrels hibernating at ambient temperatures $>0^\circ$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
and did not increase in hibernating arctic ground squirrels (12), a 1.6-fold increase in body weight (18), but 40% of the total body weight in squirrels (torpid and interbout euthermic 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). Also, cold exposure of mice and rats increases the expression of Ucp1 in brown adipocytes that have infiltrated WAT pads (9, 21). Although we do not know yet whether mitochondriogenesis or brown adipocyte conversion occur in hibernating ground squirrels, WAT 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-Ucp1 transgenic mice showed both Ucp1 mRNA and immunoreactive UCP in WAT 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...
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 mRNA in animals that were required to be continuously thermogenic while housed at ambient temperatures and with different body temperatures. Normalized Ucp2 mRNA ratios were calculated as described in legend to Fig. 4 and expressed as means ± SE.

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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