Regulation of body temperature and brown adipose tissue thermogenesis by bombesin receptor subtype-3

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lateef DM, Abreu-Vieira G, Xiao C, Reitman ML. Regulation of body temperature and brown adipose tissue thermogenesis by bombesin receptor subtype-3. Am J Physiol Endocrinol Metab 306: E681–E687, 2014. First published January 22, 2014; doi:10.1152/ajpendo.00615.2013.—Bombesin receptor subtype-3 (BRS-3) regulates energy homeostasis, with Brs3 knockout (Brs3−/−) mice being hypometabolic, hypothermic, and hyperphagic and developing obesity. We now report that the reduced body temperature is more readily detected if body temperature is analyzed as a function of physical activity level and light/dark phase. Physical activity level correlated best with body temperature 4 min later. The Brs3−/− metabolic phenotype is not due to intrinsically impaired brown adipose tissue function or in the communication of sympathetic signals from the brain to brown adipose tissue, since Brs3−/− mice have intact thermogenic responses to stress, acute cold exposure, and β3-adrenergic activation, and Brs3−/− mice prefer a cooler environment. Treatment with the BRS-3 agonist MK-5046 increased brown adipose tissue temperature and body temperature in wild-type but not Brs3−/− mice. Intrahypothalamic infusion of MK-5046 increased body temperature. These data indicate that the BRS-3 regulation of body temperature is via a central mechanism, upstream of sympathetic efferents. The reduced body temperature in Brs3−/− mice is due to altered regulation of energy homeostasis affecting higher center regulation of body temperature, rather than an intrinsic defect in brown adipose tissue.

Bombesin receptor subtype-3; obesity; sympathetic nervous system; brown adipose tissue; thermoregulation; CL316243; MK-5046

OBESEITY, AN IMBALANCE BETWEEN energy intake and energy expenditure, is associated with several comorbidities, including diabetes mellitus and cardiovascular disease. Current treatments for obesity are inadequate, stimulating research to better understand the physiology of energy homeostasis and to develop safe and effective pharmacologic treatments. The study of bombesin receptor subtype-3 (BRS-3) can advance both of these objectives.

BRS-3 is a G protein-coupled receptor for which the natural ligand is unknown, and despite its name, BRS-3 has a low affinity for bombesin and related natural peptides (7, 17, 21). In addition to other locations, BRS-3 is present in the central nervous system, including the hypothalamus and caudal brainstem (10, 16, 26, 37), regions involved in the regulation of feeding, energy expenditure, and body weight (32). Genetic and pharmacologic studies demonstrate a role for BRS-3 in energy homeostasis. Brs3 knockout (Brs3−/−) mice exhibit hyperphagia, reduced metabolic rate, and obesity (18, 27), and pharmacological blockade of BRS-3 in rats increases food intake and body weight (10). Conversely, BRS-3 agonists reduced food intake, increased metabolic rate and body temperature (Tb), and reduced body weight in mice, rats, and dogs (10, 11, 22).

Currently, the mechanisms underlying Tb regulation by BRS-3 are unclear. Brown adipose tissue (BAT) is the major site of facultative thermogenesis, dissipating chemical energy via uncoupling protein 1 (UCP1), thereby generating heat and maintaining Tb (2). BAT activity is regulated by sympathetic neural input, with upstream regulation from hypothalamic, preoptic, and other brain regions (1, 23). Thus the lower Tb in Brs3−/− mice could arise from intrinsic defects at a number of sites including BAT (5), sympathetic neuronal transmission (35), or the brain sites at the core of thermal regulatory system.

We now evaluate the consequences of disrupting BRS-3 signaling on BAT. We also examine the effects of BRS-3 agonism on BAT thermogenesis and the involvement of the central nervous system. Together these approaches allow us to suggest a mechanism for how the BRS-3 system affects Tb.

MATERIALS AND METHODS

Compounds. MK-5046 (33) was generously provided by Merck Research Laboratories (Rahway, NJ), and CL316243 and lipopolysaccharide were purchased from Sigma-Aldrich (St. Louis, MO).

Mice. C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Brs3−/− mice were provided by Dr. James Battey (18) and back-crossed at least eight generations onto a C57BL/6J background. Mice were housed at ~21–22°C in a humidity-controlled environment with a 12:12-h light-dark cycle and with water and chow (NIH-07 diet) available ad libitum. All experiments used male mice, typically 12–16 wk of age. Pentobarbital sodium (80 mg/kg ip) was used for anesthesia for physiological measurements. Survival surgery anesthesia used ketamine (100 mg/kg) and xylazine (10 mg/kg ip), with Banamine analgesia (2.2 mg/kg sc daily for 3 days). At least 7 days were allowed for recovery. All animal studies were approved by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)/National Institutes of Health (NIH) Animal Care and Use Committee.

Body and interscapular brown adipose tissue temperature measurement in anesthetized mice. Mice were anesthetized (pentobarbital) and placed on a heated (35°C) table, and a thermistor probe (YSI 427; Measurement Specialties, Shrewsbury, MA) was sutured into place underneath the interscapular brown adipose tissue (iBAT) via an incision caudal to the fat pad (4). A rectal temperature probe (YSI 455; Measurement Specialties) was inserted and secured with tape. The mouse was then positioned supinely, and a tracheal catheter was inserted, allowing free movement of room air. At least 30 min were allowed for stabilization before drug infusion.
Body and iBAT temperature measurement by telemetry in ambulatory mice. Mice were anesthetized, and an IPTT-300 transponder (BioMedic Data Systems, Seafood, DE) was sutured into place under the iBAT depot. Another IPTT-300 was inserted intraperitoneally via a midline abdominal incision and sutured to the omentum. Body and iBAT temperatures were recorded with a scanner (DAS-7007; BioMedic Data Systems). To avoid confounding temperature increases due to handling stress, all readings were taken within 60 s of initially handling a mouse with at least 1 h (and typically 2 h) between serial measurements. Alternatively, Tb and activity were continuously measured by telemetry (Mini Mitter/Philips Respironics, Bend, OR) using ER4000 energizer/receivers, G2 E-mitters implanted intraperitoneally, and VitalView software with data collected each minute. The Tb vs. activity cross-correlation analysis was performed with NeuroExplorer (Nex Technologies, Madison, AL).

Ambulatory tests of thermal regulation. Temperature preference was measured by placing mice in a stainless steel pan (64 × 15 × 20 cm, length × width × height) spanning two hot/cold plates set at 20 and 45 °C. Ninety-minute sessions were conducted on two successive days, with the last 60 min of the second session used for analysis. Position was tracked with an overhead camera and video tracking software (Ethovision 9.0; Noldus, Leesburg, VA). In the cage-switch assay, stress was induced by placing a mouse in a cage previously occupied by a different male mouse (19) with Tb and activity monitored by Mini Mitter. The febrile response at 21°C ambient temperature caused by lipopolysaccharide (10 μg/kg ip) (28) was measured by Mini Mitter. Indirect calorimetry was performed with a 12-chamber Environment Controlled CLAMS (Columbus Instruments, Columbus, OH) with ad libitum access to food and water during the entire testing period.

Intravenous infusions. Mice were anesthetized and the jugular vein catheterized using PE-10 tubing. CL316243 and MK-5046 were dissolved in saline or 5% dimethylacetamide in saline, respectively, and delivered in a volume of 1 ml/kg.

Intrahypothalamic infusions. Mice were anesthetized, and cannulas (5.25 mm, 26 gauge; Plastics One, Roanoke, VA) stereotaxically were implanted bilaterally (1.34 mm posterior, 0.75 mm lateral to bregma, 4.75 mm below the surface of the skull; Ref. 8), and secured with dental cement (Parkell, Edgewood, NY). Intrahypothalamic infusions used saline as vehicle and were given via a 33-gauge internal cannula protruding 0.5 mm past the tip of the guide cannula using a syringe pump (KD Scientific, Holliston, MA) infusing 1 μl per cannula over 60 s. The infusion targets the entire hypothalamus. The cannula position was verified by postmortem histological analysis.

Statistics. Data are means ± SE. Two-way ANOVA with or without repeated measures followed by Holm-Šídák's posttest was used for comparing genotypes vs. the treatment groups. Student’s t-test was used when two groups were compared. Analyses used two-tailed P < 0.05 as statistically significant.

RESULTS

Thermal biology in Brs3−/− mice. Tb in Brs3−/− mice was slightly lower than in controls, reaching statistical significance in the light but not the dark phase; there was no difference in physical activity levels (Table 1). To better understand the relationship between physical activity and Tb, we quantified the timing of the effect of activity on Tb by looking for the lag time that gives the best correlation between activity and Tb in a cohort of wild-type C57BL/6 mice. This cross-correlation analysis revealed that increased activity causes Tb increases with a 4-min (range, 2- to 6-min) lag (Fig. 1A). Based on this result we analyzed the Brs3−/− Tb and activity data using 10-min averages. The Tb histogram showed the lower Tb in the light phase and the slight shift in Brs3−/− mice but a similar overall pattern to the controls (Fig. 1B). The activity histogram showed the expected higher level in dark than light phase with no difference between Brs3−/− and control mice (Fig. 1C). Next, we examined Tb as a function of activity and observed a clear difference between Brs3−/− and control mice, with a greater difference at lower physical activity levels (Fig. 1D).

To further understand Tb regulation in Brs3−/− mice, a number of interventions that change Tb were tested. Brs3−/− mice increased Tb normally during the first hour in response to the stress of being handled and had a comparable febrile response to lipopolysaccharide, with the hyperthermia resolving more rapidly in the Brs3−/− mice (Fig. 2A). There was a normal circadian rhythm and Tb and physical activity increase in response to the social stress of a cage switch (19) (Fig. 2B). When Brs3−/− mice were placed in a thermal gradient, they dispersed over the gradient much more widely than the controls and chose cooler environmental temperatures (Fig. 2C). The lower preferred environmental temperature was not explained by the slightly higher body weight.

Brs3−/− mice have intact, functioning BAT. We next examined BAT function. Brs3−/− mice (12–16 wk old), while of comparable body weight to controls (wild type, 22.0 ± 2.6 g and Brs3−/− 22.6 ± 0.4 g), showed increased iBAT, inguinal white adipose tissue (WAT), and gonadal WAT weights (Fig. 3A). The increased iBAT weight is likely due to increased triglyceride content as Brs3−/− mice have larger lipid droplets (Fig. 3B). Ucp1 mRNA and protein levels were similar between Brs3−/− and control mice (data not shown). Body and iBAT temperatures in overnight fasted Brs3−/− mice were reduced compared with wild-type mice (Fig. 3C). The iBAT was slightly warmer than the body core, but this was not statistically different in this experiment.

To measure thermogenic capacity, mice were treated with a maximal dose of a β3-adrenoreceptor agonist, CL316243 (2). The Brs3−/− mice showed a greater increase in Tb, energy expenditure, and fat oxidation (indicated by the reduced respiratory exchange ratio) than the control mice (Fig. 3, D–F). Thus the intrinsic thermogenic capacity in response to a β3-adrenoreceptor agonist is clearly intact in Brs3−/− mice.

To assess an integrated thermogenic response (sensory information to brain to BAT), the body and BAT temperature response to cold exposure was measured. The slight reduction in Tb was similar in Brs3−/− and control mice (Fig. 3G) as was the rise in iBAT temperature (Fig. 3H). Together, these results demonstrate that Brs3-deficient mice have functional, inducible BAT, despite their lower body and BAT temperatures, and point to a possible reduction in neural activation of BAT.

Brs3 agonist activates BAT via a central mechanism. Brs3 agonists can increase Tb (10, 22), so we next examined the effect of a peripherally administered, brain-penetrant Brs3
agonist, MK-5046 (11), on iBAT temperature, using anesthetized mice to eliminate the effect of changes in physical activity. Mice were treated with MK-5046, CL316243, or vehicle. MK-5046 increased iBAT temperature in wild-type but not Brs3<sup>−/−</sup> mice (Fig. 4A). In contrast, as a positive control, CL316243 increased iBAT temperature in both wild-type and Brs3<sup>−/−</sup> mice (Fig. 4, B and C). Similar results were obtained measuring rectal temperature (Fig. 4D). These data demonstrate that MK-5046 has a thermogenic effect on BAT and that it is acting via BRS-3.

To determine if the thermogenic effect of MK-5046 was centrally mediated, we measured the effect of intrahypothalamic infusion of MK-5046 on Tb. MK-5046 increased Tb by 0.42 ± 0.11°C (Fig. 5, A and B), demonstrating that MK-5046 is acting centrally to increase Tb.

**DISCUSSION**

We have investigated in detail the reduced Tb of Brs3<sup>−/−</sup> mice. The results show no intrinsic defects in BAT or in the ability of the central nervous system (CNS) to engage sympathetic efferents to BAT. Rather we propose that the reduced Tb is a secondary effect of altered energy homeostasis affecting higher center regulation of Tb, as discussed below. Peripherally administered BRS-3 agonists increase Tb and BAT temperature, and small doses administered directly to the hypothalamic infusion of MK-5046 on Tb. MK-5046 increased Tb by
amus increase Tb, suggesting that CNS BRS-3 contributes to sympathetic outflow to BAT.

Intact control of Tb in Brs3<sup>−/−</sup> mice. The Tb reduction in Brs3<sup>−/−</sup> mice studied at room temperature is small, averaging ~0.34 °C below control mice. Tb differences of this magnitude are difficult to detect, so experimental manipulations are often used to increase their size. For example, in Brs3<sup>−/−</sup> mice fasting amplifies the Tb reduction (22). However, such manipulations have other effects on physiology, confounding interpretation of the results. Here we measured Tb by telemetry and analyzed Tb as a function of physical activity level and light/dark phase. This approach permits monitoring in the home cage without potentially confounding manipulations, makes use of the full dataset, and markedly increases the ability to detect Tb changes.

To our knowledge, Tb kinetics after physical activity have not been reported previously in mice. While the quantitative details are likely to depend on the level and type of activity and methods of Tb and activity measurement, only a brief lag time is expected due to the small body size and thus rapid heat loss. We observed a 4-min lag between activity and the Tb response.

The energy cost of defending Tb is ~2.0 kcal·day<sup>−1</sup>·°C<sup>−1</sup> in 27 g mice (Abreu-Vieira G, unpublished observations). Thus the ~0.34°C Tb reduction in Brs3<sup>−/−</sup> mice saves ~0.70 kcal/day. It is not known how this energy savings partitions to fat storage vs. reduced food intake. There are no reports charac-

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**Fig. 3.** Intact BAT function in Brs3<sup>−/−</sup> mice. WT and Brs3<sup>−/−</sup> (KO) mice were studied at 12–16 wk of age. A: interscapular brown adipose tissue (iBAT), inguinal white adipose tissue (iWAT), and gonadal WAT (gWAT) weights. B: histology of iBAT. Scale bar = 50 μm. C: ambulatory telemetry (BioMedic) BAT and body temperature at 9 am after a 16-h fast. D–F: effect of CL316243 on body temperature (D), energy expenditure (E), and respiratory exchange ratio (RER; F). Mice (body weight: WT, 25.0 ± 0.4 g and KO, 26.5 ± 0.8 g) were acclimatized to the indirect calorimetry chambers at 22°C for 4 days and then at 30°C for one day with vehicle or CL316243 treatment (0.1 mg/kg ip) on the 6th or 7th day in a crossover design. Body temperature (G) and difference in temperature between BAT and core body temperature upon exposure to 4°C (H). Time is time since onset of cold exposure. Data are means ± SE; n = 5–6/group. *P < 0.05.
terizing $\text{Brs3}^{-/}$ mice housed chronically at thermoneutrality, which would erase their Tb reduction, and it is unknown if thermoneutrality would exacerbate the increased $\text{Brs3}^{-/}$ adiposity, as it does in other mouse models (3, 25, 31).

What is the mechanistic basis for the Tb lowering in $\text{Brs3}^{-/}$ mice? Direct stimulation of the thermogenesis effector tissues, WAT and BAT, with a β3 adrenergic agonist demonstrated full functionality. In fact, the increases in metabolic rate and Tb for the augmented WAT and BAT, with a mice? Direct stimulation of the thermogenesis effector tissues, Brs3 and data are means ± SE; $n = 6$/group. *$P < 0.05$.

**Fig. 4.** MK-5046 increases iBAT and body temperature. Effects of intravenous treatments were studied in pentobarbital-anesthetized WT and $\text{Brs3}^{-/}$ (KO) mice at 12–16 wk of age. The change in iBAT temperature in response to MK-5046 [MK; 1 mg/kg iv, equivalent exposure to 10 mg/kg orally (33), which causes a maximal body temperature increase (11); A) and CL316243 [CL; 0.1 mg/kg iv; B], compared with vehicle (Veh) in WT and $\text{Brs3}^{-/}$ (KO) mice. For visual clarity, the vehicle data are repeated in A and B. C: mean change in BAT temperature, average of the 5- to 30-min data from A and B. Baseline iBAT temperatures were 35.2 ± 0.3 (WT Veh), 35.8 ± 0.2 (WT CL), 35.0 ± 0.3 (WT MK), 35.0 ± 0.2 (KO Veh), 34.9 ± 0.3 (KO CL), and 35.9 ± 0.2°C (KO MK). D: mean change in body temperature, average of the 5–30 min data from the same experiment. Data are means ± SE; $n = 6$/group. *$P < 0.05$.

**Fig. 5.** Effect of bilateral intrahypothalamic MK-5046 treatment (1 μg × 2) in pentobarbital-anesthetized C57BL/6 mice. Body temperature change from time baseline (A) and average of the 5–30 min data (B). Baseline temperatures were 35.7 ± 0.2 (Veh) and 35.3 ± 0.1°C (MK). Experiment is a crossover design and data are means ± SE; $n = 9$/group. *$P < 0.05$.
thetic mechanism of action (10, 22). Here, we directly show that the Tb increase occurs via BAT activation. Intrahypothalamic injection of MK-5046 increased Tb, demonstrating a role for CNS BRS-3. BAT thermogenesis is controlled at multiple levels in the CNS. Thermal sensory inputs, some routed via the lateral parabrachial nucleus (LPB), and other signals are integrated in the preoptic area, transmitted to the dorsomedial hypothalamus (DMH), rostral raphe pallidus, and on to preganglionic sympathetic neurons (23). Other regions contributing to regulating sympathetic tone to BAT include the paraventricular hypothalamus (PVH), orexinergic lateral hypothalamus (LH), and the nucleus of the solitary tract (NTS). BRS-3 binding activity (10) is found in many of these areas (e.g., DMH, PVH, LH, LPB, and NTS) as is BRS-3 mRNA (e.g., medial and median preoptic area, DMH, PVH, and LPB) (37). Further studies (such as injection of smaller volumes of agonist and localized ablation of Brs3) will be required to localize more precisely which neurons and nuclei are driving the Tb increase and their upstream and downstream interactions.

**BRS-3 agonism for the treatment of obesity?** How does mechanistic understanding of BRS-3’s role in thermal biology inform the prospects of BRS-3 agonism for the treatment of obesity? First, it is important to recognize that smaller homeotherms such as mice have larger surface area to volume ratios, generate much heat in BAT, and strive to conserve body heat (9). In contrast large homeotherms such as adult humans get most (but not all; Ref. 24) of their heat from BAT-independent metabolism and have developed mechanisms to facilitate heat loss. Due to these physiological differences, mice, unlike adult humans, vary their target Tb greatly in response to environmental changes such as cold exposure or food deprivation. As noted, the effects of the BRS-3 system on Tb are likely secondary to its primary role in energy homeostasis. This makes the concern that BRS-3 agonists will produce clinical hyperthermia unlikely, and is consistent with the lack of observed Tb changes in men treated with MK-5046 (30). The realization that adult humans can and do expend energy via BAT activation has brought attention to BAT (24) and highlighted the potential for drugs that activate BAT as a treatment for obesity (36). BRS-3 agonists fit this paradigm; they increase energy expenditure by BAT. BRS-3 agonists also inhibit food intake, which is crucial since increased food intake often accompanies BAT activation and thereby impairs weight loss due to BAT activation. However, caution is warranted. Leptin is another ligand that reduces food intake and activates BAT, and leptin is a near-miraculous drug when given to treat leptin deficiency (6). However, >99.9% of obese people have a high leptin level, not a deficiency, and treatment with leptin in these patients is not effective (13). Until an endogenous BRS-3 ligand is identified, it is unknown if obese people have low or high BRS-3 ligand levels and this may determine how effective a BRS-3 agonist will be for treating obesity.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


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