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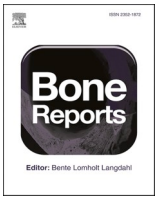

Brown adipose tissue but not tibia exhibits a dramatic response to acute reduction in environmental temperature in growing male mice

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ABSTRACT

Mice are typically housed at room temperature (~22 °C), which is well below their thermoneutral zone and results in cold stress. Chronic cold stress leads to increased adaptive thermogenesis and reductions in cancellous bone volume and bone marrow adipose tissue mass in long bones of growing mice. There is strong evidence that increased neuronal activity initiates the metabolic response of intrascapular brown adipose tissue (BAT) to cold stress, but it is less clear whether bone is regulated through a similar mechanism. Therefore, we compared the short-term response of BAT and whole tibia to a reduction in environmental temperature. To accomplish this, we transferred a group of 6-week-old male mice from 32 °C to 22 °C housing and sacrificed the mice 24 h later. Age-matched controls were maintained at 32 °C. We then evaluated expression levels of a panel of genes related to adipocyte differentiation and fat metabolism in BAT and tibia, and a panel of genes related to bone metabolism in tibia. The decrease in housing temperature resulted in changes in expression levels for 47/86 genes related to adipocyte differentiation and fat metabolism in BAT, including 9-fold and 17-fold increases in *Ucp1* and *Dio2*, respectively. In contrast, only 1/86 genes related to adipocyte differentiation and fat metabolism and 4/84 genes related to bone metabolism were differentially expressed in tibia. These findings suggest that bone, although innervated with sensory and sympathetic neurons, does not respond as rapidly as BAT to changes in environmental temperature.

1. Introduction

Adaptive thermogenesis, including shivering and non-shivering thermogenesis, is an important survival mechanism that is utilized by mammals whenever the environmental temperature falls below thermoneutrality (Silva, 2011). Brown adipose tissue (BAT) contributes to non-shivering thermogenesis (Lowell and Spiegelman, 2000). Sustained exposure to a cool environment causes increased mitochondrial activity in BAT with induction of beta oxidation of fatty acids, increased electron transport activity, and increased uncoupling protein 1 (UCP-1) levels, which together result in generation of heat (Cannon and Nedergaard, 2004).

Thermoneutrality refers to the environmental temperature at which an animal does not have to generate or lose heat to maintain core body temperature. The thermoneutral point varies in C57BL/6 J mice in relationship to photoperiod from ~29 to ~34 °C (Skop et al., 2020). However, mice are typically housed at room temperature (~22 °C), resulting in chronic cold stress (Brzek et al., 2022; van der Stelt et al., 2017). Cold stress in turn results in adaptive responses, including increased non-shivering thermogenesis (Zhao et al., 2022). Collateral effects accompanying chronic cold stress adaptation in growing male and female mice include hyperphagia, reduced white adipose tissue (WAT) stores and bone- and bone-compartment specific deficits in bone accrual and/or bone loss (Iwaniec et al., 2016; Martin et al., 2019). The mechanisms mediating the negative impact of chronic cold stress on bone metabolism during room temperature housing have received limited attention. Compared to mice housed at thermoneutral, bone formation in mice housed at room temperature is reduced and bone resorption is either unchanged or increased. Thus, at the cellular level, bone loss associated with chronic cold stress appears to be due to a negative bone turnover balance where bone resorption exceeds bone formation. At the gene level, housing temperature influenced expression levels for genes associated with osteoblast number and activity. Specifically, expression levels for the bone formation makers alkaline

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phosphatase (*Alpl*), osteocalcin (*Bglap*), and type 1 collagen (*Col1a2*) were higher in mice housed at thermoneutral temperature than mice housed at room temperature. Additionally, there were temperature-associated differences in expression levels for genes for transcription factors and genes associated with hormone receptor signaling, growth factor, and cytokine signaling (Iwaniec et al., 2016). The extent of these changes suggest that environmental temperature has large, complex effects on bone metabolism, but they do not reveal the precise mechanisms for these changes.

Lowering housing temperature from thermoneutral to room temperature increases gene expression levels for uncoupling protein 1 (*Ucp1*) in BAT by approximately 5-fold, a change associated with a dramatic increase in food consumption (Johnson et al., 2023). BAT expression of *Ucp1* is regulated by sympathetic outflow from the hypothalamus and sympathetic outflow is increased in response to cold stress (Li et al., 2019). This is likely relevant because increased sympathetic signaling is reported to be negatively associated with bone mass in rodents and humans (Kim et al., 2018; Ng and Chin, 2021; Roshanzamir et al., 2016).

Since bone is innervated (Hill and Elde, 1988, 1991), it is plausible that the skeleton can respond to sympathetic outflow in parallel with BAT. Alternatively, a distinct mechanism may be responsible. If there is commonality in the responses of BAT and bone to cold temperature stress, we would anticipate a similar time course. However, studies to date have evaluated only long-term skeletal responses to cold stress. To address this gap in knowledge, we compared the acute (24 h) response of BAT and tibia to reduction in housing temperature from 32 °C to 22 °C.

2. Materials and methods

2.1. Experimental design

The experimental protocol was approved by the Oregon State University Animal Care and Use Committee. Mice were maintained in accordance with the NIH Guide for the Care and the Use of Laboratory Animals. Four-week-old male WBB6F1 mice were obtained from Jackson Laboratory (Bar Harbor, ME). The WBB6F1 mice were generated by mating WB/ReJ Kit^{W}/J (Stock No. 000692) females with C57BL/6J-$Kit^{W\text{-}v}$/J (Stock No. 000049) males. WBB6F1 mice serve as wild type controls for double heterozygote ($Kit^{W/W\text{-}v}$) mice. $Kit^{W/W\text{-}v}$ mice have been extensively used for immunological research. $Kit^{W/W\text{-}v}$ mice are also of interest to the bone community for understanding bone/adipose/immune cell interactions as these mice exhibit skeletal phenotypes related to their macrocytic anemia, mast cell deficiency, and failure to accrue fat in long bones and lumbar vertebra (Iwaniec and Turner, 2013; Turner et al., 2011). Importantly, the anemia and mast cell deficiency, but not failure to form bone marrow adipocytes, are rescued by adoptive transfer of purified hematopoietic stem cells isolated from WBB6F1 mice (Deyhle Jr. et al., 2019). WBB6F1 mice can be derived by breeding parental strains. Following arrival, the mice were housed individually at 32 °C for 2 weeks. At 6 weeks of age, the mice were randomized into one of two groups (n = 6/group): (1) continued at 32 °C or (2) transferred to 22 °C. The mice were euthanized 24 h later and interscapular BAT and tibia collected for downstream gene expression analyses. BAT was placed in RNALater (ThermoFisher Scientific, Waltham, MA) and tibiae were flash frozen in liquid nitrogen. Both tissues were stored at −80 °C until analysis. Food (Teklad 8604, Harlen Laboratories, Indianapolis, IN) and water were provided ad libitum for the duration of study and all animals were maintained on a 12 h light/dark cycle.

2.2. Gene expression

Whole tibiae were pulverized with a mortar and pestle in liquid nitrogen. Total RNA was isolated from BAT and tibia following homogenization in Trizol reagent, and mRNA was reverse transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix for quantitative polymerase chain reaction (qRT-PCR) (ThermoFisher Scientific). qPCR was done using Fast SYBR Green Master mix (ThermoFisher Scientific). Expression of a panel of genes related to adipocyte differentiation and fat metabolism was determined using Mouse Adipogenesis RT2 Profiler PCR array (PAMM-049ZA) (Qiagen, Valencia, CA). Expression of a panel of genes related bone metabolism in tibia was determined using Mouse Osteogenesis RT2 Profiler PCR array (PAMM-026ZA) (Qiagen). PCR array gene expression was normalized to *Gapdh*, *Gusb*, and *Hsp90* housekeeping genes. Relative quantification and statistical analyses for PCR array data were done using GeneGlobe RT2 Profiler PCR Data Analysis Portal (Qiagen). *Adrb1* and *Adrb3* gene expression were determined using predesigned *Adrb1*- and *Adrb3*-specific KiCqStart™ Primers (MilliporeSigma, St. Louis, MO). Data represent averaged fold-regulation in differentially expressed genes in response to transfer of mice from thermoneutral (32 °C) to room temperature (22 °C) housing.

3. Results

The treatment groups were well matched for body weight, with mice in the 32 °C group weighing 24.2 ± 0.8 g and mice in the 32 °C to 22 °C group weighing 23.6 ± 0.6 g (mean ± SE, n = 6/group).

As shown in Fig. 1A, transferring mice from 32 °C to 22 °C resulted in numerous changes in expression of a panel of genes related to adipocyte differentiation and fat metabolism in BAT. In total, 47/86 genes were significantly differentially expressed, with 27 genes up-regulated and 20 genes down-regulated. Genes experiencing large increases (>2.5-fold) in expression included *Cebpb*, *Dio2*, *Fasn*, and *Ucp1*. Genes experiencing large decreases (>2.5-fold) in expression include *Adrb2*, *Jun*, and *Lep*. *Adrb1* expression was increased (2-fold) in BAT of mice transferred from 32 °C to 22 °C. There was a trend (P = 0.053) for an increase (1.6-fold) in *Adrb3* (data not shown).

As shown in Fig. 1B, transferring mice from 32 °C to 22 °C resulted in negligible change in tibia in expression of the same panel of genes related to adipocyte differentiation and fat metabolism as used for analyzing BAT. Specifically, only 1/86 genes (*Agt*) was differentially expressed. Furthermore, only 4/84 genes related to bone metabolism (*Bmp2*, *Bmp2r*, *Tfbr3*, *Vegfa*) were differentially expressed in tibia in response to treatment (Fig. 1C). None of the differentially expressed genes in bone approached a 2.5-fold change. A complete list of genes evaluated can be found in Supplemental Tables 1 and 2. As noted in the tables, only a small number of genes were expressed at levels where detection was unreliable (defined as Ct > 30).

4. Discussion

We investigated the 24-hour response of BAT and tibia to cold temperature stress induced by transferring male mice from near thermoneutral (32 °C) to room temperature (22 °C). To ascertain this short-term response, we evaluated a panel of genes related to adipocyte differentiation and fat metabolism in BAT and tibia and a panel of genes related to bone metabolism in tibia. Transfer of mice from thermoneutral housing to room temperature housing had a major impact on gene expression in BAT, whereas few changes were detected in tibia.

Cold challenge studies in mice are often performed by reducing ambient temperature from room temperature (~22 °C) to as low as 4 °C and are typically of long duration (Hadadi et al., 2022; Lelis Carvalho et al., 2021; Yau et al., 2021). Cold exposure is known to activate adrenergic signaling in BAT to initiate thermogenesis (Silva, 2011). Primary cultures of brown adipocytes are stimulated by treatment with the adrenergic receptor agonist norepinephrine suggesting that this is a direct central nervous system to BAT action (Bianco et al., 1992). In support, we observed dramatic changes in expression of genes related to adipocyte differentiation and fat metabolism in BAT within 24 h following transfer from thermoneutral housing to room temperature housing. Moreover, the acute reduction in environmental temperature in the present study resulted in increased gene expression levels in BAT

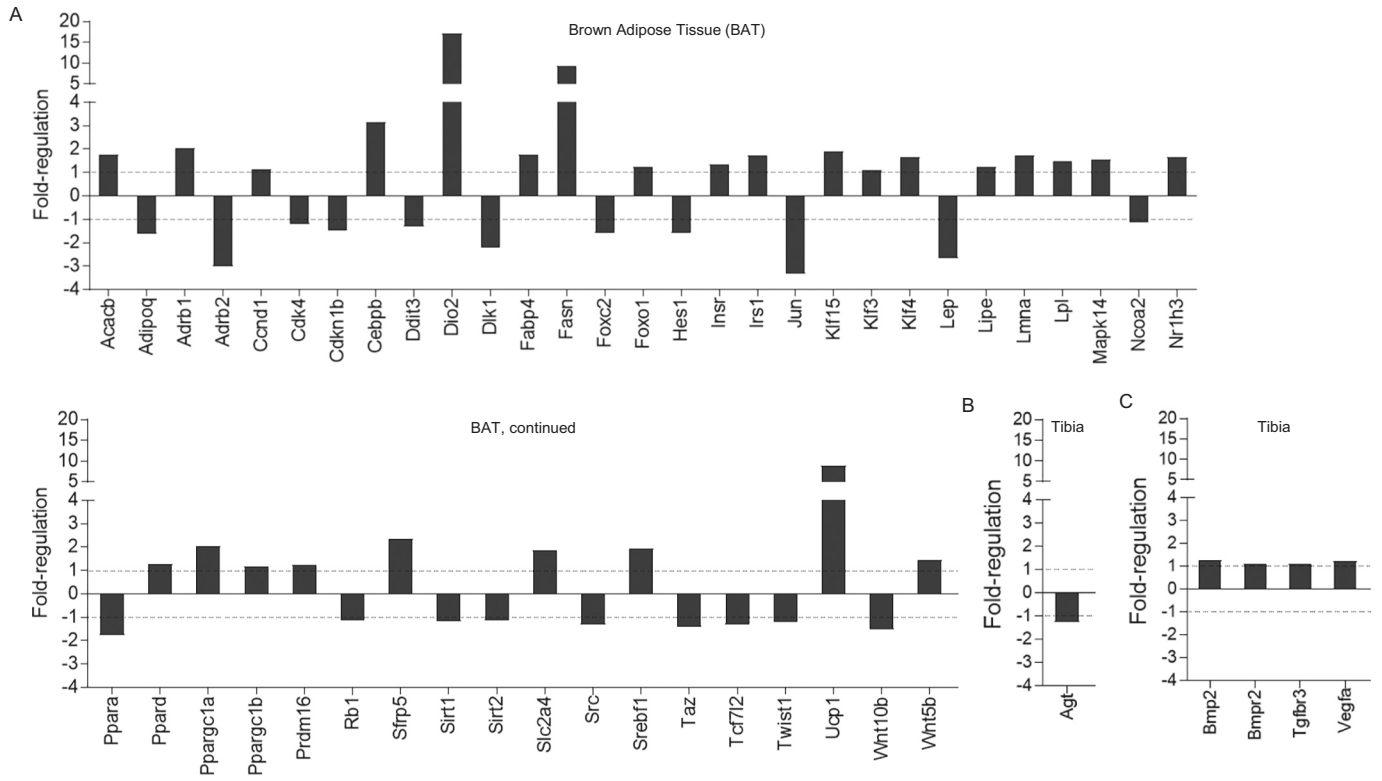

Fig. 1. Differential expression of genes related to adipocyte differentiation and fat metabolism in BAT (A), adipocyte differentiation and fat metabolism in whole tibia (B), and bone metabolism in whole tibia (C). Data represent averaged fold-regulation (compared to mice housed at 32 °C) in differentially expressed genes ($P < 0.05$), with values greater than one (upper dotted line) indicating up-regulation and values less than one (lower dotted line) indicating down-regulation in response to transfer of mice from thermoneutral (32 °C) to room temperature (22 °C) housing.

for key enzymes mediating lipid metabolism, including acetyl coenzyme A carboxylase beta (*Acacb*), fatty acid binding protein (*Fabp4*), fatty acid synthase (*Fasn*), hormone sensitive lipase (*Lipe*), and lipoprotein lipase (*Lpl*). Expression levels of insulin receptor (*Insr*) and insulin receptor substrate 1 (*Irs1*) were also higher while leptin (*Lep*) and adiponectin (*Adipoq*) were lower. Lower *Lep* expression is interpreted as reflecting a reduction in lipid stores. Adiponectin is reported to inhibit adaptive thermogenesis (Qiao et al., 2014). Thus, lower expression level for *Adipoq* is anticipated with increased thermogenesis. Taken together, these findings are consistent with an early increase in intracellular lipolysis and lipid turnover associated with activation of brown adipocytes following exposure of mice to a cool environment (Khedoe et al., 2015).

Following short-duration cold exposure, we noted large increases in expression levels for *Dio2* (17-fold) and *Ucp1* (9-fold) in BAT. In agreement, Yau et al. (2021) reported that mRNA levels of *Dio2* and *Ucp1* were elevated after 72 h of cold exposure. *Dio2* encodes type 2 iodothyronine deiodinase (D2), the enzyme that coverts inactive thyroxine (T4) to the active form of thyroid hormone, 3,3′,5-triiodothyronine (T3). D2 is believed to be very important for adaptive thermogenesis in BAT (de Jesus et al., 2001). Specifically, intracellularly generated T3 appears to be necessary to saturate thyroid receptor α binding sites, which in turn initiates the signaling pathway mediating the sympathetic response of brown adipocytes. The large increase in *Ucp1* expression observed in this study, and typically observed following longer duration cold exposure, provides evidence that increased lipid metabolism supports heat generation in mice through protein uncoupling in the mitochondrial electron transport system.

Agt, which encodes angiotensinogen, was the only detected differentially expressed gene related to adipocyte differentiation and fat metabolism in tibia 24 h following transfer of mice from thermoneutral to room temperature housing. *Agt* plays an important role in lipid accumulation by adipocytes (Carroll et al., 2013). A reduction in *Agt* expression could reflect the lower bone marrow adipose tissue (BMAT) observed in mice following long duration housing at room temperature but an early change is surprising. Twenty-seven genes, including *Agt*, were differentially expressed in tibia when mice were housed for 14 weeks at 32 °C compared to room temperature-housed mice (Iwaniec et al., 2016). Of these 27 genes, 16 overlap (11 in the same direction) with differentially expressed genes in BAT detected 24 h following transfer from thermoneutral to room temperature housing. The overlap may be related to the negative impact cold stress has on lipid storage in fat depots, including BAT, BMAT and WAT (Iwaniec et al., 2016).

Similar temperature-related changes in gene expression in BAT were reported in another experiment (Turner et al., 2020). We interpret these findings as evidence for significant overlap between the skeletal and BAT response to chronic cold stress. However, there were also notable differences between BAT and tibia. In contrast to BAT, neither acute nor chronic cold stress increased expression levels of *Dio2* or *Ucp1* in tibia (Iwaniec et al., 2016; Turner et al., 2020). Furthermore, we did not observe strong evidence for a cold-induced increase in fatty acid turnover in tibia.

Bone is innervated with sensory and sympathetic neurons (Hill and Elde, 1991) and β1- and β2-adrenergic receptors are located on osteoblasts (Huang et al., 2009). Neonatal sympathectomy and sensory denervation following capsaicin treatment were shown to alter bone response, in opposing directions, in a rat model for induced bone remodeling (Hill et al., 1991). Sensory neurons expressing calcitonin gene related peptide play a role in adaptive thermogenesis (Makwana et al., 2021). Also, β2-adrenergic receptor deficiency results in increased calcified cartilage thickness and subchondral bone remodeling (Rosch et al., 2021). Treatment with low dose epinephrine accelerates osteoblast differentiation (Uemura et al., 2010) and β-adrenergic receptor agonists can stimulate bone resorption in organ culture (Moore et al.,

1993). Finally, we have shown that the nonspecific β-adrenergic receptor antagonist propranolol blocks the effects of temperature stress on WAT and BMAT. However, propranolol does not antagonize the increase in *Ucp1* expression in BAT following cold exposure (Turner et al., 2020). Taken together, these findings suggest that neuronal signaling contributes to skeletal adaptation to chronic cold stress. However, they do not inform whether the action on bone is direct or indirect.

Only 4 genes related to bone metabolism were differentially expressed in tibia 24 h following transfer from thermoneutral to room temperature housing. The expression levels of each of these genes (*Bmp2*, *Bmp2r*, *Tfbr3*, *Vegfa*) increased following 24 h of housing at room temperature. This compares to 42 genes that were differentially expressed after 14 weeks (Iwaniec et al., 2016). *Bmp2r* and *Vegfa*, but not *BMP2* and *Tfbr3*, were found to be differentially expressed in the longer duration study but changes were in the opposite direction. Taken together, our findings suggest that acute changes in environmental temperature do not directly influence bone metabolism.

There are multiple plausible mechanisms for an indirect role for β-adrenergic signaling in mediating the skeletal response to cold stress. These include changes in the circulating levels of leptin and T3. Increased non-shivering thermogenesis induced by cold stress results in depletion of lipid stores in WAT resulting in decreased leptin levels. This may be important because leptin is required for optimal bone accrual in growing mice (Philbrick et al., 2017). Specifically, leptin deficiency results in decreased bone formation. As noted, room temperature housing results in (1) increased central nervous system activity and (2) increased D2-mediated conversion of inactive T4 to active T3. Increased sympathetic activation of β-adrenergic receptors stimulates UCP-1-mediated thermogenesis in BAT (via adrenergic receptor β3) and triglyceride release from WAT (via adrenergic receptors β1 and β2). High levels of D2 and absence of conversion to inactive 3,5-diiodo-L-thyronine (T2) by thyroxine 5-deiodinase (D3) in BAT leads to increased circulating levels of T3, which can enter osteoblasts via the thyroid hormone transporter MCT8. Chronically elevated T3 levels increase bone resorption, resulting in bone loss and increased fracture risk (El Hadidy el et al., 2011; Sawin et al., 1989; Vestergaard and Mosekilde, 2002).

This study has potential limitations. We cannot rule out the possibility that our gene profile did not include key early response genes regulated by environmental temperature in bone that in turn would lead to changes in bone metabolism. This seems unlikely because other regulatory factors (e.g., sex steroids, parathyroid hormone) influence expression of structural proteins in 24 h (Westerlind et al., 1995). Evaluating gene expression in whole tibia may not detect potential regional changes in response to treatment. However, the method used in this study allows direct comparisons with earlier investigations where we detected robust skeletal effects of housing temperature on gene expression (Iwaniec et al., 2016). Our study was performed in growing male mice. Cold stress induces adaptive thermogenesis and premature cancellous bone loss in both males and females. Although very few temperature by sex interactions in skeletal response to housing temperature have been identified to date (Martin et al., 2019), additional studies will be required to confirm that our failure to detect acute effects of temperature change on bone can be generalized to female mice. Our study utilized WBB6F1 mice which are derived by breeding parental strains. However, we have shown that WBB6F1 mice respond to room temperature cold stress, ovariectomy, and simulated microgravity as other mouse strains, including the commonly used B6 mice (Deyhle Jr. et al., 2019; Iwaniec and Turner, 2013; Keune et al., 2017; Turner et al., 2011; Turner et al., 2018).

In summary, in contrast to BAT, minimal changes in expression of a panel of genes related to adipocyte differentiation and fat metabolism were detected in whole tibia 24 h following transfer of mice from 32 °C to 22 °C. Long-term exposure to cold stress results in differential expression of numerous genes related to fat and bone metabolism in tibia, bone loss and decreased BMAT. Taken together, these findings suggest that the mechanisms underlying the metabolic responses of BAT and bone to cold stress differ. Therefore, while bone metabolism in mice is profoundly influenced by chronic cold stress, the skeleton appears to be insensitive to acute changes in environmental temperature.

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CRediT authorship contribution statement

Carmen P. Wong: Writing – review & editing, Validation, Investigation, Formal analysis, Conceptualization. **Urszula T. Iwaniec:** Writing – review & editing, Conceptualization. **Russell T. Turner:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bonr.2023.101706.

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