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Uncoupling protein-1 is protective of bone mass under mild cold stress conditions

Amy D. Nguyen [a,1], Nicola J. Lee [a,1], Natalie K.Y. Wee [b], Lei Zhang [a], Ronaldo F. Enriquez [b], Ee Cheng Khor [b], Tao Nie [e], Donghai Wu [e], Amanda Sainsbury [a,c,1], Paul A. Baldock [b,1], Herbert Herzog [a,d,*,1]

[a] Neuroscience Division, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, Sydney, NSW 2010, Australia
[b] Osteoporosis and Bone Biology Division, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, Sydney, NSW 2010, Australia
[c] The Boden Institute of Obesity, Nutrition, Exercise & Eating Disorders, Sydney Medical School, The University of Sydney, Sydney, NSW 2006, Australia
[d] Faculty of Medicine, University of NSW, Kensington, Sydney, NSW 2052, Australia
[e] Key Laboratory of Regenerative Biology, Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510663, China

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ABSTRACT

Brown adipose tissue (BAT), largely controlled by the sympathetic nervous system (SNS), has the ability to dissipate energy in the form of heat through the actions of uncoupling protein-1 (UCP-1), thereby critically influencing energy expenditure. Besides BAT, the SNS also strongly influences bone, and recent studies have demonstrated a positive correlation between BAT activity and bone mass, albeit the interactions between BAT and bone remain unclear. Here we show that UCP-1 is critical for protecting bone mass in mice under conditions of permanent mild cold stress for this species (22 °C). UCP-1$^{-/-}$ mice housed at 22 °C showed significantly lower cancellous bone mass, with lower trabecular number and thickness, a lower bone formation rate and mineralising surface, but unaltered osteoclast number, compared to wild type mice housed at the same temperature. UCP-1$^{-/-}$ mice also displayed shorter femurs than wild types, with smaller cortical periosteal and endocortical perimeters. Importantly, these altered bone phenotypes were not observed when UCP-1$^{-/-}$ and wild type mice were housed in thermo-neutral conditions (29 °C), indicating a UCP-1 dependent support of bone mass and bone formation at the lower temperature. Furthermore, at 22 °C UCP-1$^{-/-}$ mice showed elevated hypothalamic expression of neuropeptide Y (NPY) relative to wild type, which is consistent with the lower bone formation and mass of UCP-1$^{-/-}$ mice at 22 °C caused by the catabolic effects of hypothalamic NPY-induced SNS modulation. The results from this study suggest that during mild cold stress, when BAT-dependent thermogenesis is required, UCP-1 activity exerts a protective effect on bone mass possibly through alterations in central NPY pathways known to regulate SNS activity.

Introduction

Brown adipose tissue (BAT) has a primary function to generate heat by dissipating energy through the process of non-shivering thermogenesis. BAT is thus energy-utilising, and enhanced BAT activity reduces energy balance and may help to combat obesity. This is in contrast to white adipose tissue (WAT), which is primarily involved in the storage of energy as lipids, with its excess accumulation leading to obesity. In recent years, interest in the functions of BAT has increased markedly following the demonstration that the presence of BAT is not restricted to rodents or infants, but is also present in considerable amounts in adult humans [1–3]. Whilst BAT in mice is generally specific to the scapular region, in humans BAT is found in the neck, supraclavicular, paravertebral and suprarenal regions [1]. Importantly, research has shown a certain plasticity of fat tissue leading to a phenomenon called 'browning' of white adipose tissue depots or skeletal muscle, resulting in 'beige' or 'brite' ('brown in white') adipocytes [4,5]. Browning occurs during events such as cold-exposure and strength training [4,6]. Interestingly, browning is also associated with skeletal-related events such as heterotopic ossification [7] or triggered by direct injection of bone morphogenetic protein 7 [8]. This ability to induce brown adipocytes, or BAT-like depots, has sparked great research interest because the induction of brown adipocytes could be employed as a potential obesity treatment. Our understanding of the regulatory process surrounding BAT activity however is incomplete, and more research is required to determine the pathways involved in BAT thermogenesis and how altered BAT activity influences other tissues, including skeletal tissue.

Recently it has emerged that the regulation of whole body energy homeostasis is closely linked to the control of bone metabolism. Brown adipocytes together with white adipocytes, myocytes, chondrocytes and osteoblasts share the same mesenchymal stem cell

* Corresponding author at: Neuroscience Division, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, Sydney, NSW 2010, Australia. Fax: +61 2 9295 8281.
E-mail address: h.herzog@garvan.org.au (H. Herzog).
[1] These authors contributed equally to this work.

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precursor [9]. Interestingly, rats undergoing cold exposure, when BAT is highly activated, exhibit reductions in circulating concentrations of bone formation markers, indicating that cold stress may reduce osteoblast function [10]. Furthermore, BAT activity has been demonstrated to positively correlate with bone mass [11,12]. In one of these studies, a model of impaired BAT function, the *Misty* mouse, was employed. This mouse model exhibited decreased cancellous and cortical bone mass, which resulted from elevated sympathetic tone [12]. On the corollary, a mouse model of induced brown adipocyte growth exhibited anabolic effects on the skeleton *via* release of paracrine factors which affected bone remodelling [11]. Human studies have also demonstrated a positive link between BAT activity and bone mass in children [13] and young, non-obese women [14], but these studies are correlative and cannot ascertain causality. These findings are of great interest given recent research showing that BAT function decreases in adult humans from approximately 50–60 years of age [15–17], an age where bone dysfunction is increasingly common [18]. This research is in its relatively fundamental stages, however, and the BAT–bone relationship has not yet been studied in detail.

The ability of BAT to dissipate energy in the form of heat occurs specifically through the actions of uncoupling protein-1 (UCP-1). Various studies in rodents have looked at BAT contributions to energy homeostasis, either in models of BAT ablation [19], cold stress [20], at thermo-neutrality [21], and through transgenic overexpression [22–24] or disruption of BAT function [21,25]. However, the exact details of UCP-1's effect upon bone homeostasis have not been determined. These details are important as they may form part of an indirect regulatory pathway between BAT and bone. Several studies have corroborated that defects in BAT function or UCP-1 ablation resulted in obesity [19,21,26,27]. However, other studies have demonstrated that BAT-defective or UCP-1 ablation mouse models, although having impaired thermogenesis, may be resistant to diet-induced obesity [25, 28–31]. These conflicting data demonstrate that the metabolic, as well as the skeletal, phenotypes of UCP-1$^{-/-}$ mice remain to be clearly elucidated. Thus several critical aspects of the BAT–bone relationship remain to be clarified: 1. What is the component of BAT that regulates bone? 2. What is the pathway by which BAT activity alters bone metabolism? Resolution of these issues would provide novel information regarding this emerging anabolic pathway to bone, and would also increase our understanding of the complex interactions between skeletal and energy homeostasis, an increasingly important issue in contemporary society.

In order to address these questions we analysed the skeletal phenotype of our novel UCP-1$^{-/-}$ mouse model, which unlike previously published models [21,25] is based on a point mutation that renders the UCP-1 protein inactive. We specifically examined the role of UCP-1 in controlling bone mass and metabolic phenotypes at temperatures of thermo-neutrality (29 °C), which does not require activation of BAT UCP-1, as well as under conditions of mild cold stress (22 °C, room temperature), when BAT UCP-1 activation is required for temperature control, but has not yet reached a level of cold stress in which the mice would require employment of shivering thermogenesis [32]. Importantly, this is also the temperature of standard housing across many animal research facilities, thus responses to this mild cold stress relative to thermo-neutrality will be broadly applicable to many murine studies.

Materials and methods

Ethics statement and animal care

All research and animal care procedures were approved by the Garvan Institute/St Vincent's Hospital Animal Ethics Committee and are in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All mice were group housed (3–5 per cage), except for short periods of time to enable food intake and indirect calorimetry measurements, under conditions of controlled temperature (22 °C or 29 °C) and illumination (12 h light–dark cycle, lights on at 7:00 h) and were given soft bedding and tissues for nesting as well as a dome to hide under for environmental enrichment. Mice were given *ad libitum* access to water and standard chow (8% calories from fat, 21% calories from protein, 71% calories from carbohydrates and 2.6 kcal g^{-1}; Gordon's Specialty Stock Feeds, Yanderra, New South Wales, Australia), unless otherwise stated. Male mice were used throughout.

Generation of knockout mice

A spontaneous point mutation in mice on a C57BL/6 background resulted in changing a Cytosine nucleotide into an Adenosine nucleotide at position 39 in exon V of the UCP-1 gene. This change leads to the generation of an alternative splice-acceptor side that is preferentially used and as a consequence this modification causes the deletion of 13 amino acids from the UCP-1 mRNA, rendering the resultant protein inactive and undetectable even in a truncated form (Fig. 1A). Mice carrying this mutation were crossed with C57/BL6 mice four times to reduce the risk of other mutations also being carried forward. Breeding of heterozygous mice ensured the generation of wild type (WT) littermates which were used as controls.

The UCP-1 luciferase reporter knockin mouse was generated similarly to that previously published [33] by targeting the firefly luciferase coding sequence into the last coding exon of the mouse UCP-1 gene by homologous recombination in mouse ES cells. The resulting knockin luciferase was brought under the control of the endogenous UCP-1 transcriptional unit. The confirmed ES clones were injected into the blastocysts and germline transmitted positive mice were identified.

Cold stress intervention

Prior to the thermo-neutral intervention, all mice were housed at 22 °C, as per standard housing conditions of laboratory rodents. Male WT and UCP-1$^{-/-}$ mice from 7 weeks of age were then either housed at temperatures of thermo-neutrality (29 °C) or maintained at mild cold stress conditions of 22 °C for 10 weeks.

Western blot

To confirm the successful ablation of UCP-1 from our UCP-1$^{-/-}$ mice, we conducted a Western blot on protein extracted from the brown adipose tissue (BAT) of both UCP-1$^{-/-}$ and WT mice. BAT samples taken from animals were homogenised in RIPA buffer (25 mM Tris·HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche, Germany). After centrifugation, clear lysates were collected and protein concentrations were measured by a microplate spectrophotometer (Molecular Devices Inc., CA). Equal amounts of tissue lysates (20 μg protein) were resolved by SDS-PAGE and immunoblotted with antibodies against UCP-1 (Alpha Diagnostic International Inc., Texas), using GAPDH (Cell Signalling Technology, MA) as a positive control. Immunolabelled bands were then visualised or quantified using densitometry.

Bone micro-computed tomography (micro-CT)

Following fixation, right femora were cleaned of muscle and analyses were carried out using micro computed tomography (micro-CT) with a Skyscan 1172 scanner and associated analysis software (Skyscan, Aartselaar, Belgium), as previously described [34]. The X-ray source was set at 50 kV and 200 mA, with pixel size of 4.37 μm. The image slices were reconstructed using NRecon (Skyscan). Reconstruction was carried out with automated misalignment compensation for each individual sample. The reconstructed images were then straightened using Dataviewer software (Skyscan). Cancellous bone of the distal femur was selected for analysis by drawing a region of interest, starting at

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