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Measurement of Interscapular Brown Adipose Tissue of Mice in Differentially Housed Temperatures by Chemical-Shift-Encoded Water-Fat Magnetic Resonance Imaging

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Abstract

Purpose—To determine differences in fat-signal fraction (FF) from chemical-shift-encoded water-fat MRI of interscapular BAT in mice housed at different ambient temperatures (T_a).

Materials and Methods—C57BL/6J male mice (8 weeks old) were singly housed at 16°C, 23°C or 30°C ($n=16$ /group) for 4 weeks. Measures included food intake, body weight (both measured weekly) and body composition (at baseline, 2 and 4 weeks post-thermal exposure); chemical-shift-encoded water-fat MRI was performed on a 9.4 Tesla Bruker magnet with respiratory gating and anesthesia at 4 weeks post-thermal exposure.

Results—A significant inverse relationship between food intake and T_a was evidenced ($p < 0.0001$). Lean mass was similar between groups, while total fat mass was significantly different between groups ([mean $\pm$ SE]: 30°C=5.10 $\pm$ 0.19 g; 23°C=4.18 $\pm$ 0.16 g; 16°C=3.48 $\pm$ 0.54 g; $p < 0.0001$). Mean BAT-FF was positively related to T_a (means: 30°C=79.4%; 23°C=61.8%; 16°C=50.9%; $p < 0.0001$).

Conclusion—These cross-sectional results demonstrate that MRI measurement of FF within the interscapular BAT in mice reflects recent functional status of the tissue, with a lower T_a leading to a significantly reduced BAT-FF, indicative of the tissue's involvement in thermogenesis.

Keywords

thermogenesis; white adipose tissue; brown adipose tissue; water-fat MRI; fat-signal fraction; body composition

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INTRODUCTION

The study of brown adipose tissue (BAT) and its role in thermogenesis and body weight regulation has been largely limited to small animal models, due to technical limitations for quantifying and characterizing the activity of the tissue *in vivo* (1). In rodents, the interscapular BAT depot is the largest depot, located on the dorsal side of the animal between the shoulder blades, although multiple, distinct sites of BAT depots are shared between mammalian species (1). In contrast with the highly efficient energy storage capacity of white adipose tissue (WAT), BAT can consume significant amounts of energy in the process of thermogenesis. This is accomplished with numerous mitochondria that contain the uncoupling protein 1 (UCP1), which uncouples adenosine triphosphate (ATP) production from substrate oxidation (1), resulting in the consumption of energetic substrates and the generation of heat. Interest in the physiological role of BAT in adult humans goes back over three decades (2), with recent increased interest surrounding positron emission tomography/computed tomography (PET/CT) reports showing metabolically active depots in adult humans (3–9), confirming previously described anatomical depots for infants and adults (10–14). However, there exists a need for multiple modalities to safely and non-invasively quantify and characterize BAT *in vivo* particularly for longitudinal, repeated scanning.

Previous reports have utilized chemical-shift-encoded water-fat magnetic resonance imaging (WFI) applying the IDEAL (Iterative Decomposition with Echo Asymmetry and Least squares estimation) technique for the identification and quantification of interscapular BAT depots *in situ* in mice based on the measured fat-signal fraction (FF) (15). BAT, which has intermediate FF values (approximately equal quantities of lipid and water components), can be distinguished from both WAT (which exhibits high FFs due to the large amount of lipid and low water content) and lean tissue (which exhibits low FFs values due to low lipid and high water content). Beyond the presence or amount of BAT, the activation state of BAT plays a significant role in energy balance and is highly regulated to function in body temperature homeostasis and diet-induced thermogenesis in rodents (1).

It is known that reducing ambient temperature (T_a) results in an acute increase in energy expenditure in rodents, reflecting increases in shivering and non-shivering thermogenesis (1). With prolonged exposure, reduced T_a provides a chronic stimulation of the sympathetic nervous system response, causing a number of compensatory changes to conserve heat by reduced thermal loss and increased thermogenesis (1). In mice, the interscapular BAT depot (and other BAT depots) significantly contributes to the nonshivering thermogenic response, demonstrating a number of cellular and tissue level alterations (16). These alterations include an increase in the mitochondrial content (including UCP1), vascular supply and a reduced abundance of intracellular lipid (1, 16). In this work we assess whether WFI measured FF values can reflect differences in functional states of interscapular BAT depots. It was hypothesized that housing mice at reduced T_a which is known to increase thermogenic demand and stimulate non-shivering thermogenesis through BAT, would result in cellular and tissue changes in interscapular BAT (e.g. lower lipid content) that would be measured and quantified by WFI as a lower FF.

MATERIALS AND METHODS

Animal care, food intake, body weight and body composition

C57BL/6J male mice (6 weeks old) were acclimated to our animal facility for two weeks and subsequently singly housed in environmental chambers (Powers Scientific, Pipersville, PA, USA) at either 16°C, 23°C or 30°C T_a ($n=16$ /group, referred to as CT-cool temperature, RT-room temperature and HT-high temperature). Standard housing temperature for rodent

facilities is $\sim 23^{\circ}\text{C}$, while the lower limit of thermoneutral temperature for mice is $\sim 30^{\circ}\text{C}$ (17). To produce a “dose response” in ambient temperature exposure, a subsequent $\sim 7^{\circ}\text{C}$ temperature reduction from “standard” conditions was also tested to represent a “cold stimulus” ($\sim 16^{\circ}\text{C}$). Chamber temperatures as measured by min-max thermometers ranged over a $1\text{--}2^{\circ}\text{C}$ window with normal measures of $\sim 16^{\circ}\text{C}$, $23\text{--}24^{\circ}\text{C}$ and $28\text{--}30^{\circ}\text{C}$ respectively. Mice were provided *ad libitum* access to a standard rodent chow (Teklad Global 16% Protein Rodent Diet, Harlan, Madison, WI, USA) and autoclaved water, with a 12:12 hour light:dark cycle. Approximately 200 grams of sterilized Beta Chip[®] bedding (NEPCO, Warrensburg, NY, USA) and one IsoBLOX[™] Enrichment Square (#6060, Harlan/Teklad, Madison, WI, USA) was provided for each cage. Body weight and food intake were recorded weekly to the nearest 0.01g. *In vivo* body composition, including total body lean and fat mass, were determined by quantitative magnetic resonance (QMR, EchoMRI 3-in-1; Echo Medical Systems, Houston, TX, USA) (18, 19) at baseline and at two and four weeks post-thermal exposure. Briefly, mice were placed in a clear plastic tube with displacement cap to maintain position within the magnetic field of the instrument. Scans were performed on unanesthetized animals taking ~ 90 seconds and quantifying total lean and fat mass based on relative proton signals from respective chemical species. All animal research was conducted in accordance with the Institutional Animal Care and Use Committee.

Water-Fat Magnetic resonance imaging

All MRI data were acquired through the Small Animal Imaging Core using a 9.4T Bruker Magnet (BioSpec; Bruker Biospin, Billerica, Mass) with a single-channel surface coil (Bruker BioSpin) as receiver. At 4 weeks post-thermal exposure, each mouse was placed individually in the prone position on an animal bed equipped with circulating warm water to regulate body temperature to $\sim 37^{\circ}\text{C}$. Mice were anesthetized with inhaled isoflurane (1–2%) for the duration of the scans. An MR compatible small-animal respiratory device (SA instrument, Stony Brook, NY) was used to reduce motion artifacts by gating the pulse sequence. To assess the fat-signal fraction of BAT, a 3-echo WFI approach based on the IDEAL technique (20, 21) was adopted (15). Upon data reconstruction, separated water and fat signals of the underlying tissues are generated on a voxel-wise basis across the imaging volume, from which a FF is computed. A 2D multi-slice spoiled gradient echo pulse sequence was used with the following imaging parameters: repetition time (TR) = 295 msec, echo times (TEs) = 2.8/3.8/4.8 msec, echo train length = 1, flip angle = 10° , field-of-view = 3×3 cm, 128×128 acquisition matrix, receiver bandwidth = 200 kHz, and number of signal averages = 6, where each k-space phase encoding line was acquired six times and averaged before proceeding to the next line acquisition. No parallel imaging acceleration was employed. At 9.4T, the chemical-shift resonance frequency difference between water and methylene fat protons is approximately 1.36kHz and an echo spacing of 1ms translated to roughly 480° phase shifts (4x the optimal water-fat phase shift of $2\pi/3$ as described in the ideal reference (31; 32). Eight contiguous axial slices were acquired in an interleaved fashion, with a slice thickness of 1 mm. Only automated first-order linear shimming of the B_0 field was performed, while manual second-order shimming was not. The imaging time for each animal was approximately 30 minutes. The MRI was reconstructed offline using Matlab software (The Mathworks, Natick, Massachusetts) and code using a multi-fat-peak spectral model for brown adipose tissue (22, 23). The reconstruction code is available from the Matlab toolbox introduced at the recent ISMRM workshop on WFI (<http://ismrm.org/workshops/FatWater12/>) (24).

Image analysis

The largest and most easily identifiable BAT depot in mice is the interscapular depot, which is located on the dorsal side, immediately inferior to the shoulder and fore limbs. Interscapular BAT depots were delineated based on anatomical location and the FFs were

calculated across the entire voxel range indicating the interscapular BAT depot within slices, with up to 8 slices covering the entire IBAT depot. Multiple regions-of-interest (ROIs) were manually drawn within the interscapular BAT (IBAT) depot on the reconstructed MRI FF maps in each animal, across all slices that exhibited the tissue (see Figure 3). An average interscapular BAT FF was then computed for each animal. In placing the ROIs, particular care was taken to avoid inclusion of any confounding signals from the subcutaneous white adipose tissue which may overlay the BAT depot.

Histological Analysis

Immediately following sacrifice, interscapular BAT was dissected and divided for histological and biochemical processing. One half was fixed in 10% neutral and isotonic buffered formalin for 24 h and then transferred and washed in ethanol before processing for paraffin embedding. 5- μ m thick sections were cut from the paraffin blocks and stained with haematoxylin and eosin (H&E), and then viewed and photographed using a digital video camera (Nikon, Japan) mounted on the microscope at X10, X20 and X40 magnification.

Western Blotting

The other half of the interscapular brown adipose tissues were snap frozen in liquid nitrogen and stored in -70°C until analysis. Frozen samples were homogenized and sonicated in an ice-cold lysis buffer (sample:buffer = 1:10) as previously described (25). Lysates were assayed for total protein content by using bicinchoninic acid technique with bovine serum albumin as a standard.

For immunoblotting, 30 μ g of total protein from the IBAT samples were loaded onto 4–12% Bis-Tris Midi Gels (Invitrogen™). Proteins were then semi-dry transferred (Trans-Blot SD, Bio-Rad laboratories) to polyvinylidene difluoride membranes and blocked for 1 h with 5% nonfat dry milk in PBST at room temperature. Membranes were incubated overnight at 4°C with either a goat polyclonal antibody to UCP1 (C-17, 1:1000, Santa Cruz Biotechnology®, Inc.) or a mouse monoclonal antibody to β -Actin (C4, 1:1000, Santa Cruz Biotechnology®, Inc.) in 1% PBST. After incubation with appropriate secondary antibodies, immunodetection analyses were accomplished using the Enhance Chemiluminescence Kit (New England Nuclear Life Science Products, Boston, MA). Protein levels were assessed by densitometry for UCP1 and β -Actin levels using ImageJ (NIMH, Bethesda, MD). The ratio of UCP1: β -Actin was used for comparisons.

Statistical analysis

Measures of food intake, body weight, body composition and IBAT FF are reported as group means and standard deviation (SD) or standard error (SE) of the mean as indicated. Comparisons of body weight, food intake, body composition, and IBAT FF between groups were performed by ANOVA with repeated measures for longitudinal outcomes and significance defined at $p < 0.05$. Post-hoc tests of between group differences were performed with Tukey's HSD. All statistical analyses used SAS 9.1 statistical software (SAS Institute, Cary, NC, USA).

RESULTS

Food intake, body weight and body composition

Housing mice at the lower T_a resulted in a significant increase in average daily and total food intake ($p < 0.0001$) (Fig. 1.a). Despite this increased intake, only final body weight was significantly different between groups at study completion ($p=0.02$), with significant differences between the lowest and highest T_a groups final body weight ($p < 0.05$; *post hoc*

analysis), with neither significantly different from normal T_a housed animals ($p > 0.05$; *post hoc* analysis) (Fig. 1.b). Fat mass (as measured by QMR) was significantly different between groups at 2 and 4 weeks post temperature exposures ($p=0.0008$; $p < 0.0001$) and greater in warmer animals at 2 and 4 week measures (4 week change in fat mass: CT=0.51g, 19%; RT=1.29g, 46%; HT=2.10g, 71%) (Fig. 1.d), while lean mass was similar among the groups during the study (Fig. 1.c; all weeks $p > 0.6$; 4 week change in lean mass: CT=0.25g, RT=0.27g, HT=0.26g).

Brown adipose tissue

The WFI measured mean BAT FF of the interscapular depots was positively related to T_a and significantly different between groups ($p < 0.0001$; [mean $\pm$ SD] CT=50.88 $\pm$ 5.59, RT=61.83 $\pm$ 6.37, HT=79.37 $\pm$ 3.78). Axial slices of the reconstructed FF images of interscapular BAT from representative mice in each of the three T_a are shown, demonstrating a decreasing FF with decreasing T_a (Fig. 2.a-c). A composite image showing 4 different mice from each T_a group is also presented (Fig. 3). Histological examination revealed more multi-locular lipid droplets with more cytoplasm in the coolest housed animals interscapular BAT (Fig. 4; 16°C group); and more uni-locular lipid droplet filled cells in the warmest animals (Fig. 4; 30°C group), supporting the measured BAT FF differences between animals and groups. Additional measures of uncoupling protein 1, assessed by western blotting, are shown in Figure 5 a & b, along with the calculated relative UCP1 level and animal specific interscapular BAT FFs. A significant inverse association between UCP1 content and BAT FF was observed across the sub-sample of animals examined for both measures (Fig. 5.c; $P < 0.0001$).

DISCUSSION

The ability to quantitatively measure the amount and activity of BAT *in vivo* is necessary to determine its relative contribution to physiologic and phenotypic outcomes including body weight regulation, body composition and obesity- or metabolic-related disease prevention. However, there is a general lack of safe, non-invasive, and therefore repeatable methods to enable longitudinal studies that can rapidly and effectively be applied to quantify BAT and assess its functional capacity. In this work, we have applied a WFI based imaging technique to measure FF *in vivo* in mice housed at different T_a . Increased non-shivering thermogenesis through BAT is a well established physiologic response to sub-thermoneutral housing in mice. Using this housing paradigm, we observe a significant reduction in WFI measured FF associated with reduced T_a . These FF measures are supported by underlying histological findings showing decreased cellular lipid content at reduced T_a , as well as an associated increased UCP1 content in animals housed at colder T_a . These results combined support the utility of WFI measured FF as a measure which reflects the histological characteristics of BAT depots under chronically altered thermal states.

Previous results applying the IDEAL MRI algorithm were successful in identifying interscapular BAT in mice based on the measured FFs (15) using a 3T human MRI system. Similarly, WFI has been utilized to identify IBAT with an animal 4.7T scanner in rats (26). While other known anatomical depots of BAT in rodents are also distinguishable based on the FF measure (intercostals, peri-renal, peri-aortic, paravertebral) (“unpublished observation”), the IBAT is the largest and most highly researched. In addition to the amount, the functional capacity of BAT is tightly regulated and a significant contributor to thermogenesis in rodents. Due to the limited quantity and disperse nature of BAT in humans, measures of functional capacity continue to be critically important for determining the relative contribution to metabolism in physiological studies.

The physiologic and metabolic response to decreased T_a is well established in rodents. An acute increase in energy expenditure, including both shivering and non-shivering thermogenesis responses, is initially evoked upon cold exposure with non-shivering thermogenesis making an increasing metabolic contribution to heat production over prolonged reduced T_a exposure (1). Previous studies have clearly shown that BAT is critical for non-shivering thermogenesis in rodents and is stimulated under conditions of reduced T_a (1). The FF measures of interscapular BAT in normally housed mouse strains (T_a of 20–24°C and the RT group in this present work) is both anatomically and numerically distinct, with measures in the current study (BAT FFs ~30–90% [min-max]) in agreement with other observations from BAT and WAT depots (WAT FFs >90%) (15, 23). As expected, reducing the T_a (16°C) resulted in a significant increase in food intake (Fig. 1.a), but with similar body weights (Fig. 1.b) to normal housing conditions (23°C) (Fig. 1.b), suggesting the additional calories consumed were being utilized for heat production rather than growth. Conversely, raising the T_a to approximate thermoneutrality for mice (30°C) significantly reduced food intake (compared with either 16°C or 23°C). Despite this energy intake reduction, both body weight and fat mass were significantly increased over the 4 week thermal exposure compared with cooler temperatures (with fat mass increasing significantly greater than the other two temperature groups), highlighting the significant impact T_a can have on metabolic and nutritional studies (17). Considering the use of rodent models for human metabolism and disease, the increased body fat observed in the animals housed at thermoneutrality (which is the temperature where humans spend the majority of their time in modern societies) emphasizes the need to consider T_a in translational study designs that are proposed to model human metabolism (17, 27, 28). Together, these results reproduce previous findings showing the metabolism of the rodent model is sensitive to T_a , with normal housing conditions causing a hyperphagic response that increases energy intake without significantly increasing body weight compared with thermoneutral housing conditions.

As interscapular BAT is a known site of non-shivering thermogenesis, alterations in T_a should stimulate a number of biochemical and molecular changes within the tissue, resulting in greater activation at decreased T_a and suppressed function/potential at elevated T_a over the time course of the study. This alteration of function is supported by the food intake and body weight gain data between groups, showing that at decreased T_a animals consumed significantly more energy but did not gain more body weight or fat mass, indicating the extra energy was utilized in thermogenesis. The changes associated with greater activation at lower T_a would include increased vascular supply, innervations, mitochondrial content and decreased lipid content, resulting in an overall lower relative FF. Conversely, elevated T_a and suppressed function should be associated with greater lipid infiltration, including the presence of WAT like cells with increased lipid stores (greater FF). As seen in Figures 4 & 5, cellular adaptations in BAT samples were dependent on the T_a supporting the ability of T_a exposure to induce changes in BAT indicative of functional status. The associated FFs measured by WFI resolved these T_a dependent changes, with a higher FF associated with the reduced need for non-shivering thermogenesis in the warmest group, and a lower FF associated with more active BAT potential in the coldest group. These results are in agreement with a recent report using multiple MRI approaches to assess functional status (water-fat fraction, as well as blood flow) in conjunction with pharmaceutical activation of BAT (29), demonstrating multiple means (ambient temperature in the present work compared with pharmaceutical agents in the recent publication) to activate BAT which result in similar histological characteristics of increased functional capacity as measured by MRI.

It is not currently possible to determine if the interscapular BAT of mice at the lowered T_a possess a greater number of smaller brown adipocytes using only WFI measures or any

other applied imaging methodology (e.g. CT, PET, etc.), as the voxel size is greater than the dimensions of a single cell (15, 30, 31). Nevertheless, it was possible to quantify the FF across a volumetric area of the interscapular depot by identifying the boundaries of the muscle composing the shoulder and back of the mouse (on the ventral and lateral surfaces of the interscapular BAT) and the dorsal subcutaneous depot of WAT that covers the interscapular BAT depot (Figs. 2&3). As seen in the water-fat reconstructed images in Figure 3, there was a consistent shift across the full FF range, demonstrating both the variability of voxel-specific FF measures within a given depot, as well as the consistent directional response of a lower FF with increased activation state (lower T_a). Previous research has shown a decreased IBAT FF in live versus dead animals (26). This work utilized live animal scans. However, anesthesia is required to immobilize the animal to achieve spatial resolution by reducing motion artifacts during the scan. It is known that anesthesia can acutely alter sympathetic nervous signaling and BAT function, which may obscure changes associate with the tissue activation state (1). However, adaptive tissue characteristics would be preserved and representative of the animals' chronic thermal state as assessed by both MRI and histology. Although a warming sled was used during the MRI scans, the T_a in the MRI room, in combination with the anesthetic effect, may have blunted the activation state, reducing blood flow, resulting in a higher measured FF. Anesthesia is not normally required for humans and rapid scan sequences clearly have a benefit in this regard. However, the benefits of respiratory gating could be applied to reduce motion artifacts with people, as motion distortion was still present in a handful of scanned animals.

We recognize the long scan time involved in this work, which was not optimal. This scan time was compounded by several factors, including the use of a single echo train, a single element coil that was not compatible with parallel imaging, six signal averages to obtain confidently high signal-to-noise ratios, and the need to perform respiratory gating. Modifications and improvements in any or all of these aspects can easily reduce the scan time per animal to a few minutes in future applications.

The results presented here represent the BAT status of mice from a given strain, at a particular age, with a single diet, singly housed, but exposed to different T_a . Differences in housing conditions (single versus group, temperature, etc.), diet (fat amount and type, protein amount, carbohydrate, etc.), age of animals, genetic background and early life exposures are just a few examples of factors that potentially influence the amount and activity of BAT (1, 32, 33). Even within a single group of experimentally controlled inbred mice, just as variability in body size and food intake are frequently observed, BAT amount and FF among individual animals can be strikingly different. This further emphasizes the need for non-destructive methods to perform *in vivo*, longitudinal measures to determine the intra-animal changes that occur in response to specific interventions, permitting pre- and post- intervention measures for each animal to serve as its own control.

Similar to other applied imaging techniques like PET/CT, MRI currently cannot directly quantify, at the molecular level, the uncoupling protein 1 (UCP1) content or tissue specific energy expenditure. Additionally, UCP1 content alone, although correlative with functional capacity, is not sufficient to determine the metabolic rate of brown adipose tissue as the uncoupling function is tightly regulated at multiple levels, demonstrated by the rapid changes in activity associated with acute thermal exposures and sympathetic activation. Thus, energy expenditure could not be accurately assessed from UCP1 content alone, even if somehow measured with an exogenous UCP1-specific molecular tag, but would require other approaches that can assess variables related to energy expenditure such as blood flow, oxygen consumption (34) and/or temperature elevation. PET/CT requires the uptake of radiolabels to study BAT, preventing an *a priori* identification and quantification of metabolically quiescent BAT depots with the technique. Nevertheless, recent results using

PET/CT have shown that the Hounsfield unit value, a measure of x-ray attenuation or tissue density, is increased in activated BAT (35, 36), suggesting retrospective analyses of quiescent depots may be possible. Considering the expense of radionuclides and increased risk from radiation exposure using PET/CT (37) additional imaging modalities are needed to study BAT in humans. These scans were performed on a dedicated small bore 9.4T animal scanner. Previous work with human clinical 3T scanners suggests similar methods can be adapted for human scans and a recent report has successfully implemented similar techniques to identify BAT in human infants (15, 38). As these results represent one live scan per animal, future studies will focus on the ability to longitudinally measure *in vivo* changes within a single animal, better controlling for individual variation in amount or function of specific BAT depots.

In conclusion, WFI measured FF provides an accurate, non-invasive measure of interscapular BAT reflecting histological changes associated with alterations in BAT functional capacity and may provide a reliable means to perform longitudinal *in vivo* studies of BAT in animals and humans.

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Figure 1.

Food intake, body weight and body composition in mice. (a) Average daily food intake (grams/mouse/day) for 4 weeks (mean $\pm$ SE). (b) Weekly body weight (grams/mouse) from baseline through 4 weeks (mean $\pm$ SE). (c) Fat mass and (d) lean mass as measured by QMR at baseline, week 2 and week 4 (mean $\pm$ SE). (Letters shared in common among the groups indicate no significant difference; $p > 0.05$).

Figure 2.

In vivo FF maps of interscapular BAT in mice. (a) Three representative axial cross-sections showing the fat images, water images and FF maps from WFI (FF color scale to right), of the dorsal interscapular BAT depot (arrows) of a representative 16°C-housed animal, (b) 23°C-housed animal and (c) 30°C-housed animal.

Figure 3.

Axial reconstructions of interscapular BAT depots from 4 different animals shown for each T_a group. Note lower FF values in the cold-stimulated animals. White adipose tissue is characteristically red (~90–95% FF) and lean muscle and tissues are purple (<10% FF).

Figure 4.

Histology of interscapular BAT. Representative interscapular BAT with H&E staining showing increased lipid content present with increasing housing temperature. Individual animals' mean MRI measured FF is CT:50%, RT:59%, HT:80%.. Individual axial slices of the MRI image is shown from the same animals in Figure 3 a, b and c.

Figure 5. UCP1 levels from interscapular BAT. (a) Western Blot showing interscapular BAT UCP1 and β -Actin levels from a representative sub-sample of animals in each of the temperature groups (CT, RT and HT). (b) Average relative UCP1 content of interscapular BAT samples (mean $\pm$ SE) (Letters shared in common among the groups indicate no significant difference; $p > 0.05$). (c) A significant, inverse relationship between UCP1 content FF (samples shown in 5.a and 5.b, ($p=0.0001$)).