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Research paper

UCP1 in adipose tissues: two steps to full browning

Anastasia V. Kalinovich, Jasper M.A. de Jong, Barbara Cannon, Jan Nedergaard*

Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden

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ABSTRACT

The possibility that brown adipose tissue thermogenesis can be recruited in order to combat the development of obesity has led to a high interest in the identification of "browning agents", i.e. agents that increase the amount and activity of UCP1 in brown and brite/beige adipose tissues. However, functional analysis of the browning process yields confusingly different results when the analysis is performed in one of two alternative steps.

Thus, in one of the steps, using cold acclimation as a potent model browning agent, we find that if the browning process is followed in mice initially housed at 21 °C (the most common procedure), there is only weak molecular evidence for increases in UCP1 gene expression or UCP1 protein abundance in classical brown adipose tissue; however, in brite/beige adipose depots, there are large increases, apparently associating functional browning with events *only* in the brite/beige tissues.

Contrastingly, in another step, if the process is followed starting with mice initially housed at 30 °C (thermoneutrality for mice, thus similar to normal human conditions), large increases in UCP1 gene expression and UCP1 protein abundance are observed in the classical brown adipose tissue depots; there is then practically no observable UCP1 gene expression in brite/beige tissues.

This apparent conundrum can be resolved when it is realized that the classical brown adipose tissue at 21 °C is already essentially fully differentiated and thus expands extensively through proliferation upon further browning induction, rather than by further enhancing cellular differentiation. When the limiting factor for thermogenesis, i.e. the total amount of UCP1 protein per depot, is analyzed, classical brown adipose tissue is by far the predominant site for the browning process, irrespective of which of the two steps is analyzed.

There are to date no published data demonstrating that alternative browning agents would selectively promote brite/beige tissues versus classical brown tissue to a higher degree than does cold acclimation. Thus, to restrict investigations to examine adipose tissue depots where only a limited part of the adaptation process occurs (i.e. the brite/beige tissues) and to use initial conditions different from the thermoneutrality normally experienced by adult humans may seriously hamper the identification of therapeutically valid browning agents. The data presented here have therefore important implications for the analysis of the potential of browning agents and the nature of human brown adipose tissue.

1. Introduction

A major undertaking presently both in the obesity field and in the thermogenesis field is to identify "browning agents", i.e. conditions or agents that would induce a "browning" response, i.e. an increase in the amount (and activity) of UCP1 in adipose tissues. Browning agents could be genetic manipulations, environmental conditions or treatment with natural or pharmaceutical agents. The purpose of these efforts would ultimately be to increase the total capacity and activity of UCP1 for combustion to help counteract the development of obesity or even to make obese persons slim. Presently, a significant – if not predominant – fraction of such scientific efforts concentrates on events occurring in the adipose tissues generally referred to as "brite" [1] or "beige" [2]. Brite/beige adipose tissue depots, such as the inguinal depot in mice, are the depots in which (a fraction of) the otherwise white-fat-like adipocytes can attain a brown-fat-like appearance and a brown-fat-like thermogenic gene expression profile. An understandable reason for this focus on the brite/beige tissues is that when

* Corresponding author. Department of Molecular Biosciences, The Wenner-Gren Institute, The Arrhenius Laboratories F3n, Stockholm University, SE-106 91 Stockholm, Sweden.
E-mail address: jan@metabol.su.se (J. Nedergaard).

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conventional molecular parameters are followed in mice, it would seem that it is in these brite/beige depots that the recruitment occurs: this seems to be where new UCP1 appears; the classical brown adipose tissue depots seem barely to react to the browning agents. In the present study, we critically examine this concept experimentally based on a physiological, integrated view.

2. Material and methods

2.1. Animals

Male C57Bl/6J mice, 12 weeks of age, were purchased from Charles River (Wilmington, USA). Upon arrival the mice were single-caged and randomly assigned to three groups of 6–8 each: 30 °C, 21 °C and 4 °C. The mice had free access to water and food (chow diet R70, Standard Diet, Lactamin AB, Sweden; 4.5 g% fat, 14.5 g% protein, 60 g% carbohydrates) and were maintained on a 12:12 h light:dark cycle (8 a.m.–8 p.m.). Mice were acclimated to different ambient temperatures for 7 weeks. During the first 4 weeks, the mice were supplied with wood chips, cardboard house, wood-wool nesting material and a wooden stick. During the last 3 weeks of acclimation, enrichment was reduced (cardboard house was removed and amount of nesting material was reduced) to ensure acclimation to the desired temperature. The mice were weighed (Supplemental Figure S1A) and were sacrificed between 10 and 12 a.m. To reduce the amount of blood in the tissues of the cold-exposed mice, they were placed at 30 °C for 15 min before sacrifice. IBAT, ingWAT and eWAT were dissected quantitatively, weighed (Supplemental Figure S1B), frozen in liquid nitrogen and stored at −80 °C until further analysis. One lobe of each tissue was used for mRNA analysis, the other lobe for protein analysis.

The experiments were approved by the Animal Ethics Committee of the North Stockholm region.

2.2. RNA isolation, cDNA synthesis, and real-time qPCR

For mRNA analysis, frozen tissues were homogenized in TRI Reagent (Sigma-Aldrich, T9424). RNA was extracted using the chloroform-isopropanol method according to the manufacturer's instructions. 500 ng RNA was reverse-transcribed using the High Capacity cDNA Kit (Life Technologies, no. 4368814) in a total volume of 20 μl. Gene-specific primers were premixed with 11 μl SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich, S4438) to a final concentration of 0.3 μM. cDNA was diluted 1:10, and aliquots of 2 μl per reaction were run in triplicate. Thermal cycling conditions were 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by melting curve analysis on a Bio-Rad CFX Connect Real-Time system. The ΔC_t method ($2^{-\Delta Ct}$) was used to calculate relative changes in mRNA abundance, i.e., C_t values for 18S rRNA were subtracted from the C_t value of UCP1 to adjust for variability in cDNA synthesis. The level of 18S rRNA per μg cDNA (RNA) was not influenced by acclimation to the 3 different environmental temperatures studied here (Supplemental Figure S1C).

Primer sequences were

for UCP1 forward 5′ – GGCCTCTACGACTCAGTCCA – 3′
and reverse 5′ – TAAGCCGGCTGAGATCTTGT– 3′;
for 18S forward 5′-AGTCCCTGCCCTTTGTACACA-3′
and reverse 5′-CGATCCGAGGGCCTCACTA-3’.

Some randomly chosen samples of extracted RNA were subjected to quality control as described in Ref. [3]. Briefly, 1 μg RNA was run in 1% agarose gel with 2% bleach (2–5% hypochlorite) and ethidium bromide at 100 mV for 20 min and detected under UV light. Three sharp bands representing ribosomal RNA (with no smearing) confirmed that the RNA samples were not degraded.

2.3. Western blots

For Western blots, one lobe of each tissue was homogenized in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA) with freshly added 1 mM Na_3VO_4, 10 mM NaF and protease inhibitor cocktail (1 tablet for 10 ml of buffer, Complete-Mini, Roche). The homogenates were centrifuged at 14,000 *g* for 15 min. The top fat layer was discarded and the lysate carefully aspirated using a 1 ml syringe and 27-G needle. The concentration of proteins in the lysate was determined using the method of Lowry. An equal volume of reducing sample buffer (62.5 mM Tris·HCl, pH 6.8, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 100 mM dithiothreitol and 0.1% (wt/vol) bromphenol blue) was added to each sample. Proteins (5 μg per lane) were separated by SDS–PAGE in ordinary 12% polyacrylamide gel (acrylamide/bis-acrylamide = 37.5/1). Proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare Life Sciences) in 48 mM Tris·HCl, 39 mM glycine, 0.037 (wt/vol) SDS and 15% (vol/vol) methanol using a semi-dry electrophoretic transfer cell (Bio-Rad Trans-Blot SD; Bio-Rad) at 1.2 mA cm^{-2} for 60 min. After transfer, the membrane was stained with Ponceau S for examination of equal loading and equal transfer to the membrane. After washing, the membrane was blocked in 5% milk in Tris-buffered saline-Tween for 1 h at room temperature and probed with UCP1 antibody at a dilution of 1:40 000 overnight at 4 °C. We verified that loading 5 μg protein from cold-acclimated interscapular brown adipose tissue does not saturate the signal when detected with the anti-UCP1 antibody diluted to 1:40 000. The UCP1 antibody had been produced in rabbit against the C-terminal UCP1 decapeptide (in-house product). The immunoblots were visualized with anti-rabbit horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Western ECL substrate, no. 170-5061, Biorad) in a charge-coupled device camera (Fuji Film). The blots were quantified using the Image Gauge V3.45 program (Fuji Film).

One IBAT sample, called “standard”, was used to compare samples on different membranes. A standard brown adipose tissue sample was loaded in two lanes on each gel and the intensities of the UCP1 bands on each membrane were normalized to the intensity of the standard sample from the same membrane. The amount of UCP1 protein in the standard sample was designated as 1 a.u. of UCP1.

2.4. Statistics

KaleidaGraph 4.5.0 was used for the graphs and Excel:Mac2011 for statistical analysis. Statistical analysis was based on Student's two-tailed *t*-test with unequal variance.

3. Results and discussion

To analyse the browning process as such, we have used the classical and still most powerful browning agent: cold. In studies aimed at identifying browning agents, these agents are given to “control” mice; we have therefore exposed mice from control conditions to cold(er) and have followed events in the classical brown and in the brite/beige adipose tissues, using the interscapular brown adipose tissue as a representative classical brown adipose tissue depot and the inguinal adipose tissue depot as a representative brite/beige adipose tissue depot. The “cold” stimulus used here should thus be seen as an example of any “browning agent”. We performed this study, starting with control mice under two different conditions: the current routine experimental set-up