



Research paper

UCP1 in adipose tissues: two steps to full browning



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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.

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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

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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 ($2^{-\Delta C_t}$) 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'-AGTCCCTGCCCTTGTACACA-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) bromophenol 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

($\approx 20^{\circ}\text{C}$), and a humanized experimental set-up (30°C).

3.1. The current routine experimental step: browning from normal temperatures

In the majority of current investigations to evaluate browning agents, mice housed at about 20°C are used. The animals are then exposed to browning agents/conditions. Here we simply use cold acclimation, i.e. the chronic transfer of mice from 21°C to 4°C , as the browning agent.

When we determine the level of gene expression in a routine experimental way after cold acclimation, we observe in the interscapular depot of classical brown adipose tissue only a modest increase in UCP1 mRNA levels as an effect of cold: slightly less than a 2-fold increase when expressed versus 18S rRNA as the reference (Fig. 1A). In contrast, we find in the brite/beige inguinal depot a very large increase, in the order of at least 10-fold (Fig. 1B).

These changes in UCP1 mRNA are principally reflected in changes in UCP1 protein. Thus, both in the representative Western blot (Fig. 2A), and in the compilation of such data from 6 mice (Fig. 2B), there was almost no observable effect of the browning agent on the level of UCP1 protein per mg tissue protein in the interscapular brown depot (some 20% increase). The situation regarding the brite/beige depot is dramatically different. Indeed, given the Western blot conditions used, it was not possible to detect UCP1 protein in the brite/beige depot in the control mice – but in the cold-acclimated mice, its presence was very marked (Fig. 2C). There was thus formally an infinite increase in the UCP1 protein level in the brite/beige depot as a consequence of the use of cold as a browning agent (Fig. 2D).

Data similar to those shown here have previously been published concerning a very broad range of browning agents. It is natural from results such as these to conclude that the browning agent works practically only on the brite/beige depots. Indeed, based on these data, it would be natural to classify only the brite/beige adipose tissue as being “recruitable” [4] or “inducible” [5] (and thus implicate the classical brown adipose tissue as being “non-recruitable” or “non-inducible”). A further conclusion would be that any metabolic effects observed at the whole animal level, such as a difference in glucose metabolism and particularly an increase in nonshivering thermogenic capacity – as is indeed observed in mice acclimated to the cold – should be solely ascribed to events in the brite/beige depots. Classical brown adipose tissue would be physiologically uninteresting for the browning process. This is a line of reasoning found in a wide range of papers published recently advocating brite/beige adipose tissue as the tissue where

the only physiologically interesting UCP1 recruitment takes place.

3.2. A more encompassing view

Thus, from the data above, cold-induced browning *apparently* only takes place in the brite/beige depots. However, this is indeed “apparent” only, because other processes occur simultaneously.

Concerning UCP1 mRNA. As seen in Fig. 3A, the total amount of RNA in the thermogenic adipose tissue depots increased as an effect of the browning agent cold. This occurs most clearly in the interscapular brown adipose tissue depot, but some increase also takes place in the brite/beige inguinal depot. (For a more complete picture, we also include data from epididymal white adipose tissue; in this depot, cold acclimation does not lead to any increase in the total amount of RNA.) Thus, even though the relative UCP1 mRNA content is $\approx$ constant (Fig. 1A), this increase in total RNA indicates in itself that there must be a significant increase in the total amount of UCP1 mRNA in the interscapular brown adipose tissue, despite the impression above of only a modest increase (Fig. 1A).

We have therefore plotted the total amount of UCP1 mRNA per interscapular brown adipose tissue depot (Fig. 3B, brown curve) (multiplication of the data in Fig. 1AB with those in Fig. 3A). In Fig. 3B, we also show the total amount of UCP1 mRNA in the brite/beige inguinal depot (the orange curve). This is now much lower (more than 10-fold lower) than the UCP1 mRNA amount in the classical brown adipose tissue. The reason for this is, of course, that the relative levels of UCP1 mRNA in brite/beige tissue at 4°C are much lower than in the classical brown adipose tissue (about 20-fold lower, compare the scales on the y-axes between Fig. 1A and B). In the epididymal adipose depot, there is so little UCP1 mRNA that it is not visible at this resolution. Thus, in contrast to the molecular data implying that major interest should focus on the events in the brite/beige depot, estimation of the total amount of UCP1 mRNA implies that most recruitment is indeed found in the classical interscapular brown adipose tissue depot.

Concerning UCP1 protein. However, UCP1 mRNA does not produce heat [6], only the UCP1 protein can be thermogenic. As seen (Fig. 3C), total tissue protein in the interscapular brown adipose tissue also increased markedly during the browning process. If these data on total mg protein in the depot are multiplied with the amount of UCP1 per mg protein (Fig. 2), we obtain the total amounts of UCP1 protein in the different depots. It is now clear that the major browning process occurs in the classical brown depot (Fig. 3D), with only a small amount of UCP1 protein being found in the brite/beige depot (about 8% of that found in the brown). Thus, in striking contrast to the impression obtained from analyzing relative

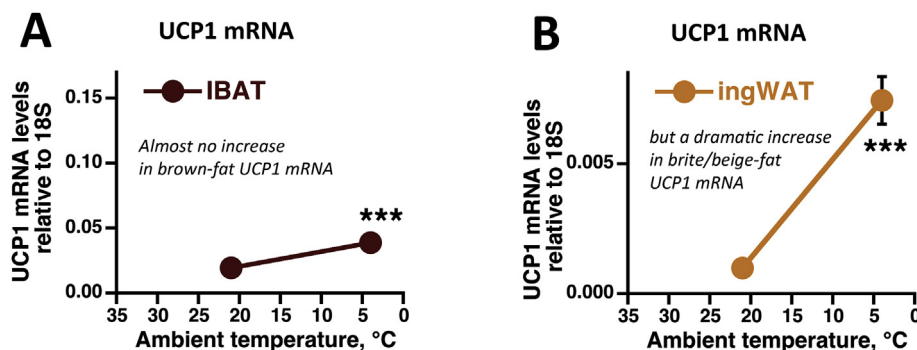


Fig. 1. Effect of standard browning procedures on UCP1 mRNA levels in different tissues. UCP1 mRNA levels (expressed per 18S rRNA) in (A) interscapular brown adipose tissue and (B) inguinal adipose tissue (brite/beige adipose tissue) under standard control (21°C) and browning (4°C) conditions. Values here and in subsequent figures are means $\pm$ SE from tissues from 6 to 8 mice; where not visible, the SE was smaller than the size of the symbol. Note the direction of the temperature change on the x-axis here and in subsequent figures. Statistics: here and in subsequent figures, *, ** and *** indicate significant effects of cold acclimation ($P < 0.05$, < 0.01 and < 0.001 , respectively).

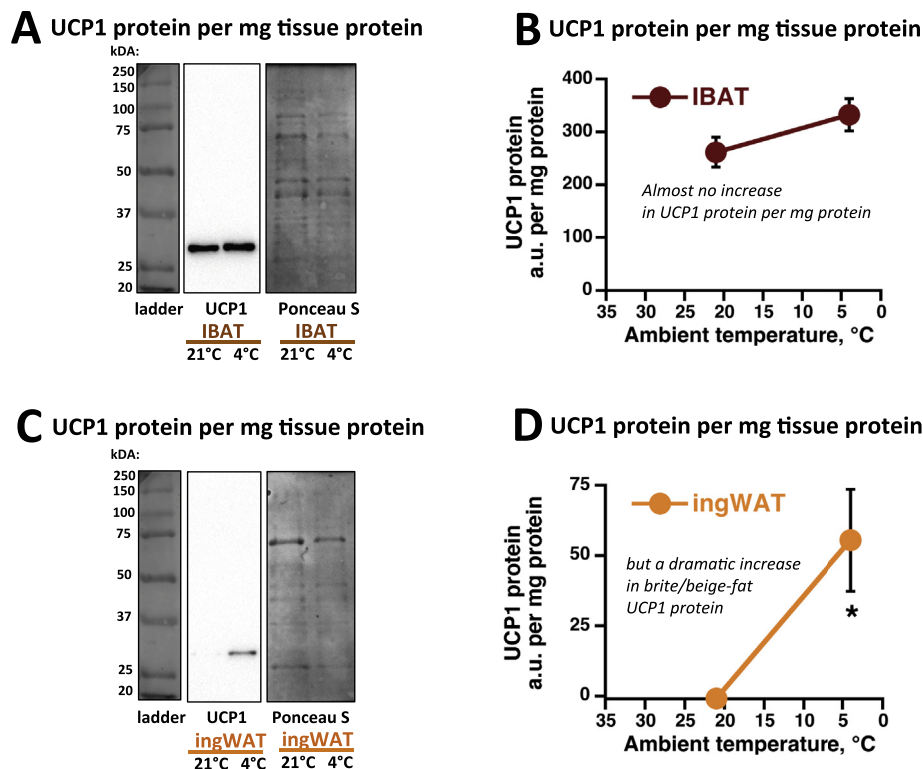


Fig. 2. Effect of standard browning procedures on UCP1 protein abundance in different tissues. A. Representative Western blots of UCP1 in lanes loaded with 5 μ g interscapular brown adipose tissue protein from control (21 °C) and browning conditions (4 °C). Right part shows Ponceau S staining of the same lanes, to demonstrate similar loading. B. Compilation of data as in A obtained from 6 mice. C and D: as A and B but with tissue protein from inguinal depots. Lanes loaded with 5 μ g inguinal adipose tissue protein. Statistics etc. as in Fig. 1.

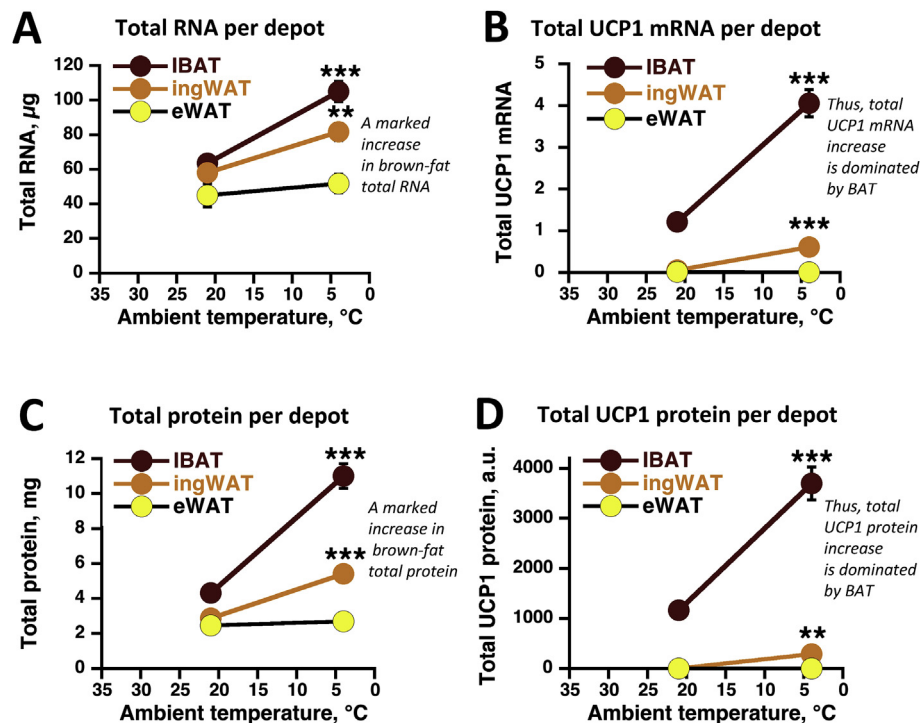


Fig. 3. Effect of standard browning procedures on the physiologically relevant parameter: total UCP1 protein amounts. A. Total amounts of RNA in the interscapular, inguinal and epididymal adipose tissue depots. B. Total amounts of UCP1 mRNA in the different adipose tissue depots. Data obtained by multiplying the data in Fig. 1AB (UCP1 mRNA per RNA) with the data in Fig. 3A (total RNA). Note that the IBAT data at 21 °C are not normalized to 1; rather, the indicated multiplication yields the value 1.199. C. Total amounts of protein in the different adipose depots. D. **The physiologically relevant parameter: the total amount of thermogenic UCP1 protein per depot.** Data obtained by multiplying the data in Fig. 2BD (UCP1 protein per mg tissue protein) with the data in Fig. 3C (total tissue protein per depot).

increases in UCP1 mRNA levels, the increase in thermogenic capacity (i.e. total UCP1 protein) observed during browning is much higher in the brown adipose tissue than in the brite/beige depot (A similar conclusion was recently published in Ref. [7].).

What is then the “correct” way to express and discuss these data? It depends. If the discussion concerns differentiation processes, the expression of UCP1 mRNA per 18S rRNA (Fig. 1) and UCP1 protein per mg tissue protein (Fig. 2) are the correct ways. However, if we are examining total thermogenic potential, as one would in a physiological or therapeutic context, the total UCP1 protein amount (the total combustion power) must be the correct parameter. This is similar to the situation that if we were to examine the total fighting power of a boxer, we would not primarily evaluate how much myosin he had per mg muscle protein; rather it would be the total amount of myosin in the muscle that we would feel in the boxer's punch.

This thus means that many conclusions presently in the literature, where physiological effects are ascribed solely to brite/beige rather than to classical brown adipose tissue, may be misleading. It may also be pointed out that if e.g. a “selective” inhibition ($\approx 90\%$) of browning of brite/beige tissue were to be achieved with practically no effect ($\approx 20\%$) on classical brown adipose tissue molecular parameters (relative UCP1 mRNA levels), this would still mean that more UCP1 was lost from the brown than from the brite/beige tissue (20% of a greater amount of UCP1 being much more than 90% of a small amount of UCP1 (Fig. 3D)). Thus, experiments where the physiological significance of brite/beige tissues has been estimated through such observations may again misleadingly indicate brite/beige tissues as the sole cause – when, in reality, it is far more likely that it is in the classical brown adipose tissue that the main effect resides.

3.3. Enhanced proliferation versus enhanced differentiation

Why are the conclusions so substantially different? A major reason is that in the mouse at 20 °C, the brown adipose tissue is already fully differentiated, i.e. per tissue unit, it cannot become significantly “better” (more thermogenic) by increasing e.g. the density of UCP1 in the tissue (as is evident from Fig. 2A). The only possibility to increase the total capacity for thermogenesis is thus to increase the number of brown adipocytes (or perhaps the total amount of protein per cell). Indeed, there is extensive evidence that browning (cold acclimation) is associated with pronounced cell proliferation activity in the brown adipose tissue, both in mice and rats [8–13], and this can clearly explain the results observed. (It is not fully clarified whether cold acclimation leads to cell proliferation in brite/beige depots as well, but probably not [13,14]). Notably, as compared to what is the case in many other tissues, cell proliferation in brown adipose tissue would seem to be stimulated via a cAMP-dependent pathway, initiated by norepinephrine [10,11,15,16], as well as through an inhibition of apoptosis, also initiated by norepinephrine [17,18]. Norepinephrine has thus the unique role in brown adipose tissue of stimulating both proliferation and differentiation, as well as function (thermogenesis). Considering the importance of cell proliferation for the acquisition of the total thermogenic capacity, this proliferation process has received remarkably little scientific attention.

3.4. The humanized step: starting from thermoneutrality

As “civilized” human beings, we spend most of our time at thermoneutrality, i.e. as a consequence of clothes and houses, our heat loss is minimized. If not physically active, we constantly display a metabolic rate close to our lowest rate of metabolism ($\approx$ “basal metabolic rate”). In contrast, mice, as used in standard

experiments such as those described above, are not at thermoneutrality when the experiment is initiated. Normal animal housing temperatures are such that the mice have to constantly increase their metabolism by some 50–60% to compensate for the high heat loss they experience at ≈ 20 °C (e.g. Ref. [19]) (in humans, this extra energy utilization would be equivalent to running some 15 km every day – or to remain, day and night, naked, without blankets, at ≈ 10 °C [20]). Thus, since the extra heat that is constantly needed in the mice maintained under standard conditions will emanate from brown adipose tissue, the 20 °C-acclimated mice already have recruited brown adipose tissue, as we indeed saw above.

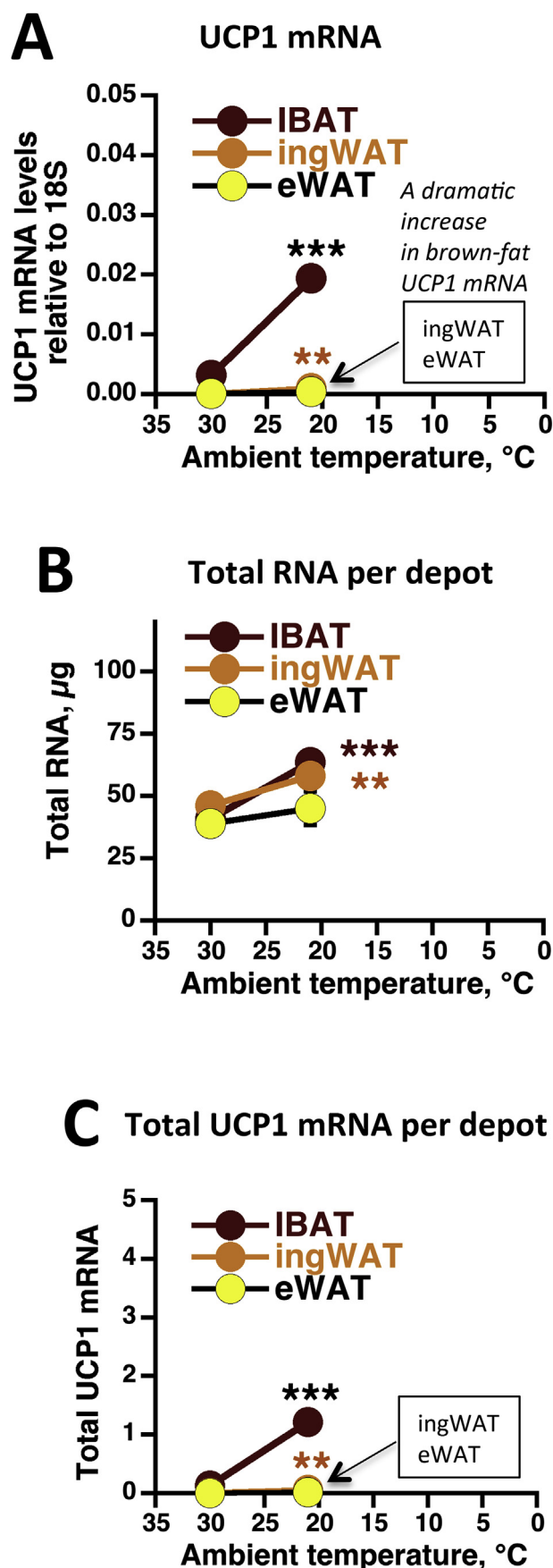
If we wish to study the effects of browning agents under conditions where mouse metabolism is somewhat more similar to ours (and we would want to do this, if we are interested in any translational predictions from our experiments), we should house the mice at their thermoneutral temperature i.e. ≈ 30 °C and study browning from this starting point. To do this has quite profound effects on the thermogenic tissues and on the interpretation of browning effects.

Concerning UCP1 mRNA. As seen in Fig. 4A, the level of UCP1 mRNA in mice living at 30 °C is very low. However, if we initiate browning in these mice by acclimating them to 21 °C, there is a dramatic increase in the level of UCP1 mRNA in the brown adipose tissue. It is occasionally possible to detect an essentially negligible increase in UCP1 mRNA in the inguinal adipose depot in these mice (Fig. 4A).

Concerning UCP1 protein. Even when mice are at thermoneutrality and (only) eating a chow diet, they possess a low amount of UCP1 in classical brown fat, that can just be discerned in the Western blot in Fig. 5A. A very marked increase in UCP1 protein per mg protein can be seen in the classical brown adipose tissue in the mice by acclimation to 21 °C (Fig. 5AB). In the brite/beige inguinal depot, practically no UCP1 protein is detectable under the conditions used here, neither on the Western nor in the compilation (Fig. 5A–D). Thus, importantly, in the browning transition from 30 to 21 °C, the classical brown adipose tissue depot displays the same molecular recruitment pattern as does the brite/beige depot in the browning transition from 21 °C to 4 °C.

The integrated view. During this browning step, there are modest increases in total tissue RNA amounts (Fig. 4B) with no significant changes in total tissue protein (Fig. 5C). Thus, calculation of the total amount of UCP1 mRNA and total UCP1 protein in classical brown adipose tissue does not dramatically alter the implications from the relative amounts and demonstrates that both UCP1 mRNA and protein are very much increased during this browning process from 30 °C to 21 °C (Figs. 4C and 5D); the levels of total brite/beige UCP1 mRNA and protein remain practically undetectable in this temperature range.

Thus, starting from the humanly relevant state of thermoneutrality, there is no indication at all of any significant involvement of brite/beige UCP1 in the acclimation step to 21 °C. This observation is pertinent for the comment that sometimes can be heard: that 30 °C-acclimated mice are not appropriate for thermogenic studies because these mice do not have brite/beige adipose tissue! Such comments are only valid if the brown(ish) adipose tissue found in humans at most of the anatomical sites of classical brown adipose tissue in mice [21] should be exclusively brite/beige in character. This has indeed been proposed [22,23] – but we find it most likely that at least some, if not most, of the brownish adipose tissue found in the neck region of adult humans is of the same nature as the classical brown adipose tissue found in these areas in mice [24,25] (our unpubl. obs.).



3.5. Two experimental steps - but a continuous adaptation

The analysis above, in which we have divided the browning process into two different “steps”, from 30 °C to 21 °C and from 21 °C to 4 °C, appears to imply that cold acclimation is not a continuous process. However, it is probably only coincidental that 21 °C appears as a break point: presumably as the acclimation temperature is decreased, the recruitment process successively shifts from primarily enhancing differentiation to subsequently promoting a tissue proliferation process. This can e.g. be seen if the data above are plotted together (Fig. 6). Thus, both UCP1 mRNA levels (per 18S rRNA), and particularly UCP1 protein levels (per tissue protein), show marked breaks when plotted in this way (Fig. 6A,D), but when we follow total UCP1 mRNA levels or total UCP1 protein levels (Fig. 6C,F), they show a linear increase from 30 °C to 4 °C, i.e. in direct proportion to the increase in heat demand occurring at these temperatures (e.g. Ref. [19]).

What can further be observed in Fig. 6, is that the increase in total UCP1 mRNA and in total UCP1 protein appear to be very similar. Indeed, when plotted together (all points where data on mRNA and protein were obtained from the same mouse), the points fall on one line (Fig. 6G), despite the data originating from different tissues (classical brown versus brite/beige) and from different acclimation conditions. Thus, under steady-state conditions, as those used here, there was a direct relationship between the amount of UCP1 mRNA and the amount of UCP1 protein (principally as earlier concluded [26]), although it should be emphasized that during a transition phase (acute temperature change etc.), this is not the case [26].

A further extrapolation from this observation, but again only valid under steady-state conditions, is that we can confidently use total UCP1 mRNA data to predict/estimate total UCP1 protein.

3.6. A global view: considering the sum of all adipose tissue depots in the mouse, practically all thermogenic power resides in the classical brown adipose tissue

All data described above originate from only two UCP1-containing depots: the interscapular classical brown adipose tissue and the inguinal brite/beige adipose tissue. Thus, it would seem possible that although the total UCP1 content in the interscapular brown adipose tissue is much higher than the amount in the inguinal fat pad (Fig. 6F), it could be that on a “global” basis (i.e. the entire mouse) the brite/beige tissues would still “win”, i.e. contain large amounts of UCP1 totally. Often it is implied that the total amounts of white (and thus possibly brite/beige) adipose tissue is very much higher than that of brown adipose tissue so that even relatively minor levels of UCP1 in these depots should sum up to large quantities of UCP1 totally. Indeed, the combined amounts of only inguinal and epididymal tissues (wet weight) are about 10-fold higher than that of interscapular brown adipose tissue (Supplemental figure S1B). Thus, combining these depots with much more white adipose tissue might point to the UCP1 located there as being metabolically very important.

We have in this study not measured UCP1 protein in all adipose tissue depots in the mouse. However, as implied from Fig. 6G, under steady state conditions we can apparently quite accurately use total UCP1 mRNA data to estimate total UCP1 protein data, and we have determined total UCP1 mRNA data in 13 different adipose tissue

Fig. 4. Effect of humanized browning procedures on UCP1 mRNA levels in different tissues. A. UCP1 mRNA levels per 18S rRNA. B. Total RNA levels. C. Total UCP1 mRNA levels per depot. The data points for 21 °C here and in the following figures are the same as those shown in Figs. 1–3.

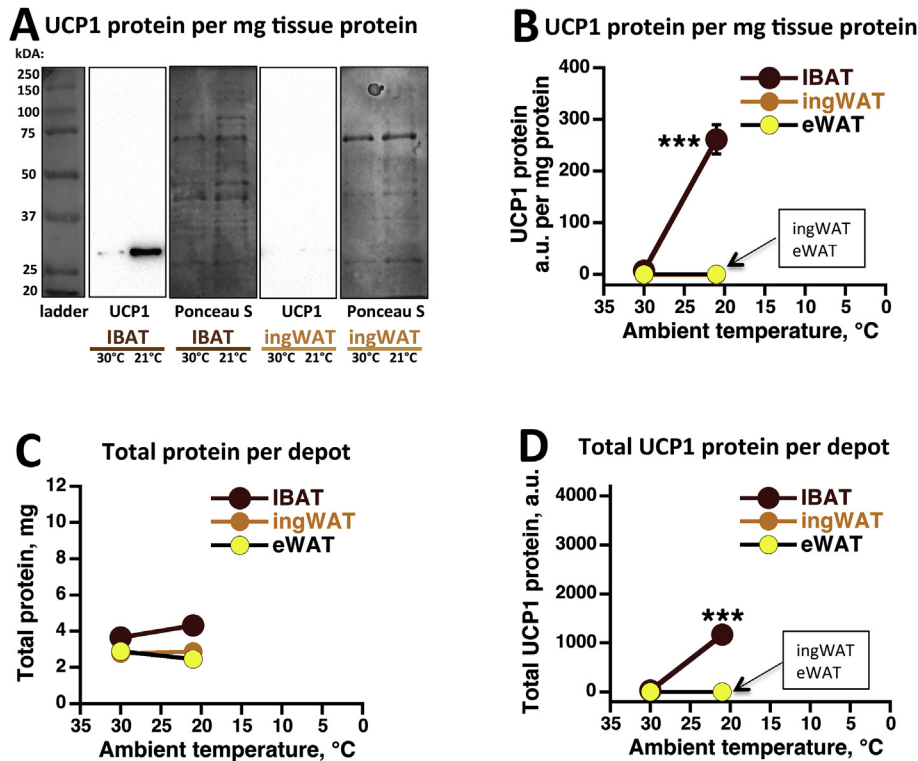


Fig. 5. Effect of humanized browning procedures on UCP1 protein levels in different tissues. A. Western blots of interscapular brown adipose tissue and inguinal adipose tissue from mice at 30 °C and 21 °C. B. Compilation of data from experiments as illustrated in A. C. Total protein amounts in different depots. D. **The physiologically relevant parameter: the total amount of UCP1 protein per depot.** Data obtained by multiplying the data in B (UCP1 protein per mg tissue protein) with the data in C (total tissue protein per depot).

depots in an earlier investigation [27]. Principally these depots include nearly all adipose tissue depots in the mouse body. Thus, we can estimate the relative global significance of brown versus brite/beige depots from their total content of UCP1 mRNA. The data are compiled in Fig. 7. As seen (Fig. 7A), the total amount of UCP1 (mRNA $\approx$ protein) is in these experiments increased 15-fold through the full scale browning by acclimating the mouse from 30 °C to 4 °C. Concerning the relative contribution of brown versus brite/beige, the picture is principally the same in both conditions: nearly all UCP1 resides in the classical brown adipose tissue depots (Fig. 7BC) although there is a somewhat higher relative contribution of brite/beige tissues at 4 °C than at 30 °C. As interscapular UCP1 mRNA amounts are less than half of all classical brown adipose tissue UCP1 mRNA and inguinal UCP1 mRNA is more than $\frac{1}{4}$ of all brite/beige UCP1 mRNA, the dominance of the classical brown adipose tissue over the brite/beige is even larger in this global view than when just the capacity of the interscapular depot versus the inguinal depot is compared, as was done in the main part of the present paper.

Thus, although use of only molecular measurements indicates that brite/beige tissue is the tissue where thermogenic capacity is recruited in the browning processes, a global physiological view clearly changes the perspectives and points to the classical brown adipose tissue as possessing the overwhelming part of the thermogenic capacity of UCP1-containing tissues.

4. Conclusions

In the present study, we have examined the browning processes in brown and brite/beige adipose tissues. We conclude that as a result of the successive recruitment firstly of increased differentiation and then of increased cell proliferation upon more intense

browning stimulation, the sole utilization of molecular markers may strongly distort our understanding of the browning processes. This distortion includes the previously proposed selective recruitment of brite/beige versus brown adipose tissues, the relative thermogenic potential of the tissues, the attribution of certain physiological effects solely to brite/beige tissues, the ability to identify browning agents, and the nature of human “brown” adipose tissue, all as discussed below.

4.1. Are brite/beige adipose tissues selectively recruited by any browning agent?

A considerable number of recently published studies on browning agents report that the browning effects are only seen in brite/beige adipose tissue depots and not in classical brown adipose tissue depots. Further scrutiny of the data show in general that this conclusion is reached by measuring basic molecular data only, i.e. relative UCP1 mRNA and protein levels, expressed per unit of RNA or protein. Such values represent a relative degree of differentiation, and as shown here, the classical brown adipose tissue in mice at ≈ 20 °C is already fully differentiated per tissue unit. Thus, even the strongest browning agent known, cold acclimation, cannot substantially increase these values in classical brown adipose tissue and, as also seen in the data presented here, it would apparently seem that the entire browning process takes place in the brite/beige tissue. Only by measuring the relevant physiological parameter, i.e. the total amount of UCP1 in the tissue, does it become clear that the increase in thermogenic capacity in the classical brown adipose tissue greatly overshadows that in the brite/beige tissues. It may be added that since measurements of total UCP1 amount have generally not been performed in the large number of reports that indicate that browning only occurs in brite/beige tissues, it cannot

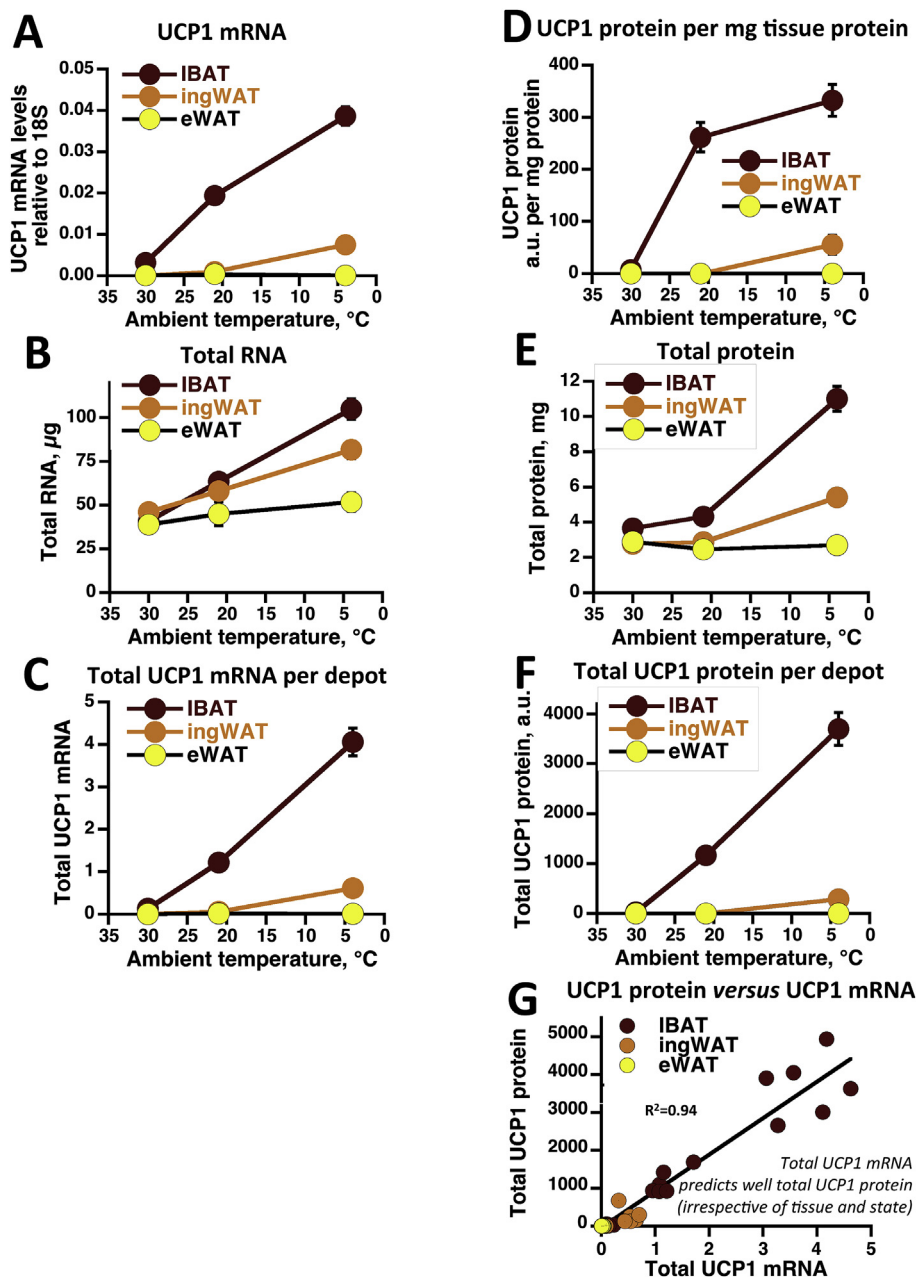


Fig. 6. An integrated view: a linear increase in thermogenic capacity with increased browning power. Data from the standard approach to browning studies (Figs. 1–3) and from the humanized approach (Figs. 4–5) are here combined to examine the coherence of the browning response. A. UCP1 mRNA per µg tissue RNA. B. Total RNA per depot. C. Total UCP1 mRNA per depot. D. UCP1 protein per mg tissue protein. E. **Total protein per depot.** F. Total UCP1 protein per depot. G. The relationship between total UCP1 mRNA levels and total UCP1 protein levels in all mice, tissues and experimental conditions. Correlation: $r^2 = 0.94$.

today be evaluated whether some browning agents may exist that indeed selectively brown the brite/beige tissues and not the classical brown adipose tissue; however, for those browning agents that work through indirect stimulation of the sympathetic nervous system (and this may be a significant fraction [28]), this would seem functionally unlikely. Consequently, until adequate measurements have been performed, observations on *selective* brite/beige browning should be viewed with some caution. Of course, it is possible to extrapolate from the data presented here and consider the relatively easily obtained observations on increased UCP1 gene expression in brite/beige as a proxy of what happens in classical brown adipose tissue. Thus, a large increase in brite/beige UCP1 mRNA may be seen as being highly indicative of a major

increase in total UCP1 protein amounts in classical brown depots (and may thus prompt measurements of this parameter).

4.2. The thermogenic capacity associated with brite/beige browning is probably only of secondary physiological importance

The data presented here are supportive of the hypothesis of the increased recruiting effects of increased sympathetic stimulation. Thus, under low sympathetic drive, only classical brown fat is addressed, leading to increased differentiation, whereas with accelerated sympathetic drive, cell proliferation in classical brown as well as differentiation of brite/beige are included. As a further consequence of the data described here, it can be concluded that,

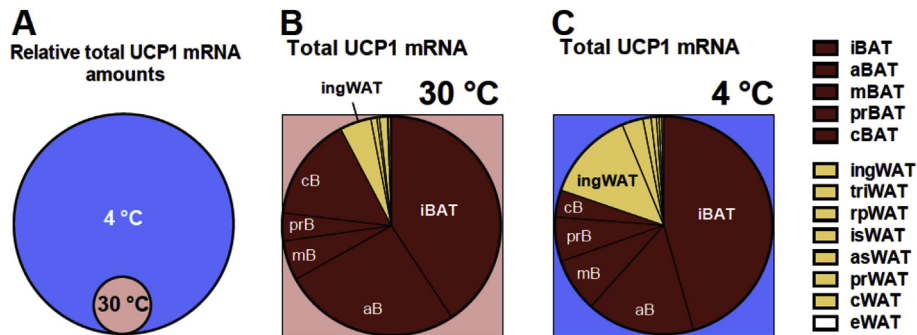


Fig. 7. An organismal view of the significance of the brown versus the brite/beige adipose tissues for the thermogenic capacity of the mouse. A. A comparison between total UCP1 mRNA levels at 30 °C and 4 °C. B. The contribution of each detectable depot to the total amount of UCP1 mRNA at 30 °C and (C) 4 °C. Note that at the state with the highest degree of browning (the most recruited state), i.e. at 4 °C, the iBAT depot followed in the previous part of the paper contains around half of the total amount of UCP1 mRNA found in the classical brown adipose tissue depots, whereas the brite/beige inguinal adipose tissue contains about ¼ of the total brite/beige UCP1 mRNA. Thus, at the organismal level, the thermogenic dominance of classical brown adipose tissue is even higher than the simple comparison between the iBAT and ingWAT depots would imply. All data in this figure are from Ref. [27] and are based on NMRI outbred mice. Due to strain differences, these NMRI data indicate more brite/beige participation than in the C57Bl/6 strain as the ability of cells in the white depots to express the UCP1 gene is probably greater in the NMRI mice than in the C57Bl/6 mice [1]. All experimental details can be found in the de Jong paper [27]. Tissue abbreviations are: iBAT, Interscapular brown adipose tissue; mBAT, Mediastinal brown adipose tissue; aBAT, Axillary brown adipose tissue; prBAT, Perirenal brown adipose tissue; cBAT, Cervical brown adipose tissue; ingWAT, Inguinal white adipose tissue; triWAT, Triceps white adipose tissue; isWAT, Interscapular white adipose tissue; prWAT, Perirenal white adipose tissue; rpWAT, Retroperitoneal white adipose tissue; cWAT, Cardiac white adipose tissue; asWAT, Anterior subcutaneous white adipose tissue; eWAT, Epididymal white adipose tissue.

under these conditions, the total capacity for heat production in the combined brite/beige tissues of the mouse is only 10–20% of the thermogenic capacity of the combined brown adipose tissues. In many published observations on brite/beige versus brown adipose tissues, the relative increase in UCP1 gene expression is the only measure given, i.e. the control level is set to 1, irrespective of the actual magnitude. The misleading impression that this gives of relative importance is illustrated in the data above, by e.g. comparing the UCP1 mRNA data in Fig. 1 (impressive increase in brite/beige, low in brown) with that in Fig. 3BD (dominating increase in brown).

The question may then be raised: why is there UCP1 expression in certain adipose tissues when the contribution to total thermogenesis is low? Important as this question is, there is no simple answer to it today. The brite/beige expression may be ascribed e.g. a local thermogenic function (but a physiological function for local heating in e.g. the inguinal depot cannot easily be motivated). Another possibility is that it is an evolutionary coincidence without marked detrimental effects and thus has been tolerated through evolution.

4.3. Physiological effects may be disproportionately ascribed to brite/beige tissues

Browning of adipose tissues is paralleled by a series of general metabolic effects, notably of course the development of non-shivering thermogenic capacity - but also other effects such as alterations (ameliorations) in blood glucose and triglyceride handling etc. It may especially be noted that if only the molecular data (UCP1 mRNA levels) from standard experimental conditions are used, it would apparently be so that the acquirement of such characteristics would be ascribed to brite/beige tissues (where relative large UCP1 mRNA changes are observed) and not to brown (where relative UCP1 mRNA changes are modest). Such conclusions have indeed been reached in the literature. However, as is understood from our data here, this argument is invalid, because the major changes in UCP1 occur in the classical brown adipose tissue depots (see Figs. 8 and 9).

Based on the realization that the brite/beige UCP1 amounts are in reality rather small, and thus that the thermogenic capacity ascribed to them may be rather small, the same type of

observations have also been interpreted to indicate that the significance of the brite/beige tissues is not related to thermogenesis - but to something else. However, no molecular explanations for such alternative mechanisms have been forwarded.

Finally, in reverse of the above argument, it has also been suggested that brite/beige depots may have special functions, based on experiments where certain treatments totally abolish brite/beige thermogenic capacity, with only minor (10–25%) effects in brown adipose tissue. If these alterations are paralleled by e.g. alterations in glucose handling, it would again be easy to be misled to conclude that only brite/beige tissues were responsible for the effects. However, calculated in absolute terms, minor alterations in brown adipose UCP1 levels may easily quantitatively overshadow major alterations in brite/beige tissues. Thus, again, studies only following relative UCP1 mRNA levels as a parameter could easily appear to imply that the large relative decrease in UCP1 mRNA in the brite/beige depots is associated with/determines the physiological parameters measured and not the small relative decrease in UCP1 mRNA in the brown depots - while the decrease in actual amounts of UCP1 protein would generally be much greater in brown than in brite/beige tissues.

4.4. Important browning agents may be overlooked

A further problem that may be encountered if only molecular markers such as relative UCP1 mRNA levels are followed during standard browning studies is that new browning agents may be overlooked. This is because such agents may not affect brite/beige but only brown tissue, and as browning agents in brown tissues do not lead to major increases in UCP1 mRNA levels, the effects of such browning agents cannot be observed. This would be disturbing because the brown tissues possess so much more thermogenic capacity.

4.5. Human brown adipose tissue may not be functionally more akin to mouse brite/beige tissue than to mouse brown adipose tissue

A topic for discussion is whether human "brown" adipose tissue is more akin to mouse brown or brite/beige adipose tissue. One argument for a closer relationship between human "brown" and mouse "brite/beige" would be whether the tissues display

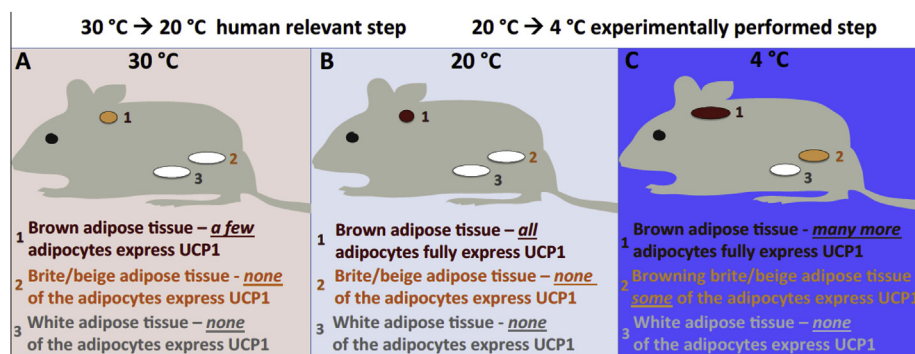


Fig. 8. Organismal principle. Most investigations use the temperate-acclimated mouse (B) as control. In this mouse, the classical brown adipose tissue depots have already achieved full differentiation (dark brown color) whereas the brite/beige depot has no UCP1 expression. When exposed to browning conditions (4 °C, C), the brown adipose tissue cannot become more differentiated (no increase in UCP1 mRNA per gram RNA or UCP1 protein per gram tissue protein). The tissue, however, expands, in the way that the relative proportion of lipid in the tissue is diminished, the protein proportion increases and more cells are formed – with a modest change in the wet weight (Supplement Fig. S1B). In contrast, if browning is followed with the thermoneutral mouse (A) as the control, the brown adipose tissue will only be partially differentiated at that temperature, and the browning process will then include an increased degree of differentiation in the brown adipose tissue (B).

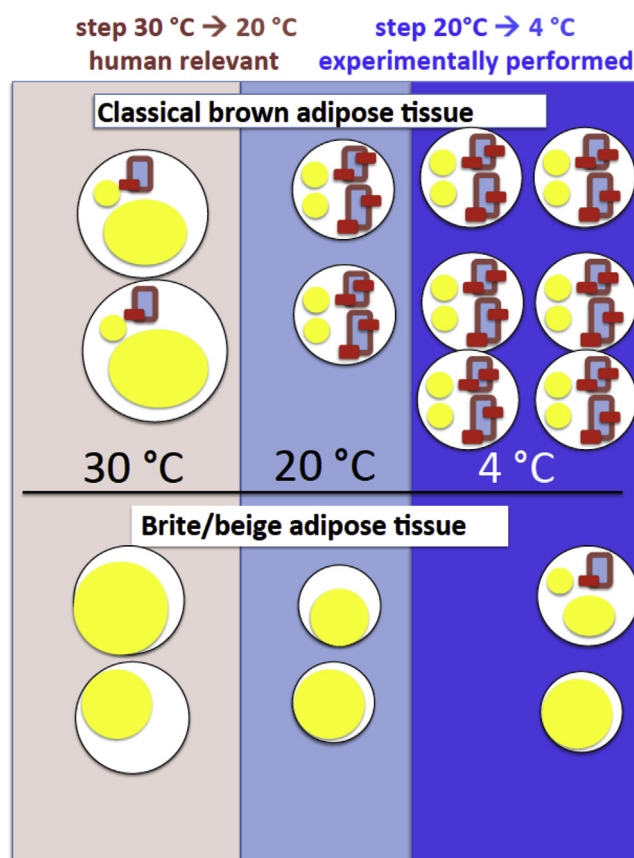


Fig. 9. Cellular principle: the two steps. Left half: the human relevant step: browning from thermoneutrality. Right half: current routine experimentation step: browning from semi-cold recruited tissues. Yellow circles: fat droplets; open rectangles: mitochondria; red rectangles: UCP1.

recruitability or not. The analysis of this is, however, not straightforward. Thus, it may be said that human “brown” adipose tissue normally displays a relatively low level of UCP1 gene expression that can be increased upon stimulation [29]. If this is compared to the mouse at 20 °C, this feature is clearly different from that of the mouse brown adipose tissue depot that displays high and not recruitable UCP1 mRNA levels (cf. Fig. 1A). Instead it is comparable

to mouse brite/beige adipose tissue that displays low but recruitable UCP1 mRNA levels (cf. Fig. 1B). Thus, it would appear that human “brown” fat displays similarities with mouse brite/beige. However, if the comparison is instead made with mice under similar physiological conditions to those pertaining to humans, i.e. at thermoneutrality, the issue becomes different. Thus, even mouse brown adipose tissue then displays low UCP1 mRNA levels and high recruitability (Fig. 4A), and no principal difference between human and mouse brown adipose tissue would then exist. However, this is only one aspect of the issue, and a further analysis of this tenet would require robust molecular markers and morphological analysis of mouse brown (and brite/beige) tissue as observed at thermoneutrality, as compared to human brown adipose tissue under the same conditions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biochi.2017.01.007>.

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