Mitochondrial Turnover

A PHENOTYPE DISTINGUISHING BROWN ADIPOCYTES FROM INTERSCAPULAR BROWN ADIPOSE TISSUE AND WHITE ADIPOSE TISSUE

Emilia Gospodarska, Pawel Nowialis, and Leslie P. Kozak

From the Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, 10-748 Olsztyn, Poland

Background: It is unknown whether there is a difference in the thermogenic potential of brown adipocytes.

Results: In iBAT, UCP1, and mitochondrial content are stable during temperature transitions, whereas in WAT they fluctuate.

Conclusion: A difference between iBAT and WAT lies in mitochondrial turnover during temperature transitions.

Significance: Brown adipocytes from iBAT or WAT may determine energy balance linked to obesity.

To determine the differences between brown adipocytes from interscapular brown tissue (iBAT) and those induced in white adipose tissue (WAT) with respect to their thermogenic capacity, we examined two essential characteristics: the dynamics of mitochondrial turnover during reversible transitions from 29 °C to 4 °C and the quantitative relationship between UCP1 and selected subunits of mitochondrial respiratory complex in the fully recruited state. To follow the kinetics of induction and involution of mitochondria, we determined the expression pattern of UCP1 and other mitochondrial proteins as well as analyzed mtDNA content after cold stimulation and reacclimation to thermoneutrality. We showed that UCP1 turnover is very different in iBAT and inguinal WAT (ingWAT); the former showed minimal changes in protein content, whereas the latter showed major changes. Similarly, in iBAT both mtDNA content and the expression of mitochondrial proteins were stable and expressed at similar levels during reversible transitions from 29 °C to 4 °C, whereas ingWAT revealed dynamic changes. Further analysis showed that in iBAT, the expression patterns for UCP1 and other mitochondrial proteins resembled each other, whereas in ingWAT, UCP1 varied ~100-fold during the transition from cold to warmth, and no other mitochondrial proteins matched UCP1. In turn, quantitative analysis of thermogenic capacity determined by estimating the proportion of UCP1 to respiratory complex components showed no significant differences between brown and brite adipocytes, suggesting similar thermogenic potentiality. Our results indicate that dynamics of brown adipocytes turnover during reversible transition from warm to cold may determine the thermogenic capacity of an individual in a changing temperature environment.

Traditionally, there are two functionally different types of adipose tissue in mammals, white (WAT)2 and interscapular brown (iBAT). WAT is primarily engaged in the storage of energy in the form of triglycerides, whereas iBAT is specialized in burning fat stores to produce heat to maintain body temperature. The uncoupling protein 1 (UCP1), located in the inner membrane of brown adipocytes mitochondria, mediates the process of thermogenesis by uncoupling oxidative phosphorylation. It has been proposed that brown adipose tissue is involved in regulation of energy balance and body fat content when an individual is in positive energy balance as a consequence of living in an obesogenic environment (1, 2). This mechanism for brown fat thermogenesis is largely based on administration of drugs and on transgenic models in which the regulation of Ucp1 expression and induction of brown adipocytes under normal physiological regulation has been disrupted (3). Recently increasing attention is being directed to the fact that brown adipocytes exist in two forms: those residing in iBAT and those in WAT of adult mice where they can be induced with a broad range of reagents and environmental conditions, thereby providing increased opportunities to regulate their thermogenic potentiality (4).

Brown adipocytes that initially arise in the fetus and form discrete depots in iBAT and those that are induced to varying extent in WAT arise from distinct developmental origins. The former cells arise from Myf5-positive progenitors that differentiate into muscle or brown adipocytes depending on the expression of PRDM16 (5). The second type of brown adipocytes found in WAT, also named “beige” or “brite” cells (6, 7), belong to a cell lineage different from classical brown cells: they first emerge in WAT as a diffuse variable population of cells between 10 and 21 days of age in rodents and then disappear spontaneously by 30 days of age but can be induced in WAT of adult animals by β-adrenergic stimulation such as cold acclimation or treatment with a β3-adrenergic receptor agonist (8). Genetic variation in the induction of brown adipocytes in WAT but not in iBAT also point to separate developmental origins for these cells (8). There are two main hypotheses concerning the developmental origin of brite adipocytes. The first one is that the white fat depots are seeded with progenitor cells that are activated and differentiate into brown adipocytes during cold exposure or other means of adrenergic stimulation (9). A second model is based upon the reversible activation of the brown adipocyte program that converts a white adipocyte to brown adipocytes.

The abbreviations used are: WAT, white adipose tissue; ingWAT, inguinal WAT; iBAT, interscapular brown tissue; UCP1, uncoupling protein 1; RC, respiratory complexes.
Thermogenic Capacity of Brown Adipocytes

adipose. Changes in the microenvironment, including the density of vascularization, the types of stromal-vascular cells in the adipocyte vicinity, and adrenergic nerve fibers, may become determining factors for a white adipocyte to differentiate into a brown adipocyte (10, 11).

Despite the similarities in the phenotype of brown adipocytes in WAT and iBAT and their expression of UCP1 protein, the mechanisms to induce brown adipocytes obviously depend on their developmental origin, as the signaling and transcription pathways as well as gene expression profiles, appear different, e.g. allelic differences at genetic loci among different inbred strains of mice control the amount of UCP1-positive cells in the white fat but have no effects in classical iBAT (8, 12–15). Therefore, it is not clear whether the thermogenic potentiality of brite adipocytes differs from that of classical brown adipocytes residing in interscapular brown fat.

To date there is little quantitative data, even in mice, concerning differences of thermogenic function of brite cells compared with classical brown adipocytes because studies of brite adipocytes are complicated by problems regarding the isolation of an enriched brown adipocyte cell population. Additionally, studies of brite adipose tissue thermogenesis have mostly focused on Ucp1 mRNA measurements, but the metabolic relevance of such expression, at least for the Ucp1 gene, is questionable (16). Comparative estimates of thermogenic capacity may be evident from the relative content of UCP1 protein to other components of the mitochondrial respiratory complex. This possibility follows from the fact that the reduced content of the F1F0-ATPase and ability to produce ATP through oxidative phosphorylation is much lower in brown fat mitochondria than in heart or muscle mitochondria (17). Because mitochondria of BAT have evolved to maximize the high levels of UCP1 for heat generation and not ATP production, the quantitative relationship of UCP1 to the respiratory components is an indicator of thermogenic potential. Thus, in this study, to evaluate the thermogenic capacity of brite versus brown adipocytes, we have used immunoblots to determine the ratio of UCP1 to selective members of the respiratory chain in mitochondria isolated from brown adipocytes of inguinal fat and classical brown adipocytes. This comparison was carried out after cold adaptation at 4 °C over a period of 15 days followed by de-induction at thermoneutrality. iBAT and ingWAT tissues were dissected and mitochondria were isolated.

We found that in mitochondria isolated from inguinal WAT (ingWAT) of cold-acclimated mice, UCP1 protein levels reached 60% that obtained in brown-fat mitochondria, but the ratio of UCP1 to the representative subunits of the respiratory chain was remarkably similar between the iBAT and ingWAT under cold conditions. Thus, the results suggest that the thermogenic capacity of brown adipocytes from iBAT and white fat depots is remarkably similar.

A second objective of this study was to determine the molecular basis for the differences in involution of brown adipocytes occurring in classical iBAT and ingWAT during reversible transitions from 29 °C to 4 °C. The experiment indicates that although the thermogenic potential of mitochondria in brown and brite adipocytes is similar, a major difference exists in determining the fate of the brown adipocyte phenotypes during the reversible transitions between thermoneutrality and the cold.

MATERIALS AND METHODS

Animals and Experimental Design—We compared the induction of Ucp1 mRNA and protein expression in ingWAT of AXB8 recombinant inbred mice, because ingWAT undergoes a facile conversion of its white adipocyte population to brown adipocytes and shows the highest levels of Ucp1 mRNA after cold stimulation (7, 13).

Breeding pairs of the AXB8 strain were fed a standard chow diet (13.2 kcal% fat, 62.1 kcal% carbohydrate, and 24.7% protein; Picolab 5053 Rodent Diet 20) ad libitum with 12-h light and dark cycles. All parental mice and their pups were kept at room temperature (23 °C). Pups were weaned at 21 days of age into the same conditions. Both male and female mice were used for cold/warm acclimation experiments.

Eight-week-old mice were singly housed and initially acclimated to a thermoneutral temperature (29 °C) for 7 days. These mice were used as controls. It should be noted that 8-week-old mice raised at an ambient temperature of 23 °C have levels of UCP1 that are very similar to those in mice maintained at 29 °C.

The Induction and Involution of Brown Adipocytes in ingWAT and iBAT—This experiment was conducted with the same animals for ingWAT and iBAT. After initial acclimation for 7 days at 29 °C, mice were maintained at 4 °C for increasing periods of time that ranged from 0.5 to 10 days and then re-acclimated to 29 °C for up to 21 days in order to stimulate their involution. Animals (4 at each time point) were sacrificed after initial acclimation and at days 0.5, 1, 2, 3, 5, 7, and 10 of cold exposure and at the days 1, 3, 5, 7, 14 and 21 of reacclimation at thermoneutrality. iBAT and ingWAT tissues were dissected immediately after killing, thoroughly rinsed with phosphate buffer to remove traces of blood and immediately frozen in liquid nitrogen, and stored at −80 °C.

The Thermogenic Capacity of Brown Adipocytes in ingWAT and iBAT—To establish the maximal level of the brown adipocyte phenotype induction in ingWAT and iBAT, mice initially acclimated at 29 °C were exposed to 4 °C, and selected animals were sacrificed after 5, 10, 15, 20, and 25 days of cold exposure. Further steps were performed as described above. To estimate the relative thermogenic capacity of brown adipocytes in iBAT and ingWAT, day 15 of cold exposure was chosen for detailed investigation, at which time UCP1 expression reached the peak. Freshly dissected iBAT and ingWAT (one pad per mouse) from four mice per time point were individually pooled, and mitochondria were isolated.

Light Microscopy—Freshly dissected iBAT and ingWAT were fixed in 10% (w/v) formalin and embedded in paraffin before sectioning. The 10-μm sections were stained with eosin/hematoxylin.

Measurement of Mitochondrial DNA Content—Mitochondrial DNA (mtDNA) content in iBAT and ingWAT was determined at three time points: after 7 days of initial acclimation at 29 °C, 10 days of cold exposure, and 14 days of reacclimation to
29 °C (five animals per group). Tissues were prepared as described previously. Total DNA was isolated by proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation. DNA was diluted to 20 ng/μl in nuclelease-free water (Sigma) and then diluted 1:10 (2 ng/μl) just before use. 5 μl of diluted DNA (10 ng) was used in a 25 μl of the reaction mixture. The relative amounts of mitochondrial and nuclear DNA were determined by real-time PCR using primers specific to mtDNA-encoded cytochrome b and to nuclear DNA-encoded cyclophilin, respectively. The PCR was performed in a Viia 7 real-time PCR system (Applied Biosystems) using the SYBR Green PCR kit (Applied Biosystems). Serial dilutions of mixed DNA from iBAT and ingWAT were analyzed in parallel to obtain a standard curve. MtDNA content was calculated from the ratio of cytochrome b to cyclophilin quantity. The primer sequences are available on request.

Isolation of Mitochondria—Freshly dissected iBAT and ingWAT were homogenized using a Teflon glass homogenizer in 10 volumes of ice-cold TES buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA) supplemented with 1 mM PMSF and protease inhibitor mixture (Sigma). All steps were performed on ice. The homogenate was centrifuged at 1000 × g for 10 min. The infranatant-containing mitochondria was transferred into a fresh tube and recentrifuged at 1000 × g for 10 min. Pellets from low speed centrifugations were resuspended in 3 volumes of TES buffer and again centrifuged at 1000 × g for 10 min. Supernatants obtained from low speed centrifugations were transferred to a clean tube and centrifuged at 10,000 × g for 10 min. The crude mitochondrial pellet was resuspended in 3 volumes of the resuspension buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA) supplemented with 1 mM PMSF and protease inhibitor cocktail (Sigma) and centrifuged at 8500 × g for 10 min. The final pellet was resuspended in 100 μl of resuspension buffer supplemented with 1 mM PMSF and protease inhibitors (Sigma). Protein concentration was measured using the Bradford reagent (Sigma). The mitochondrial suspension was stored at −80 °C.

Preparation of Tissue Lysates for Western Blotting—Frozen tissue was homogenized using a Teflon glass homogenizer in 5 volumes of ice-cold radioimmuno precipitation assay buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Nonidet P-40, 0.25% sodium deoxycholate) supplemented with 1 mM PMSF and protease inhibitor cocktail (Sigma). The homogenate was incubated on ice for 1 h and then centrifuged at 10,000 × g for 15 min at 4 °C. The clear supernatant was carefully transferred to a new tube, and protein concentration was determined by the Bradford method. The lysate was stored at −80 °C for Western blot analysis.

Western Blotting—Proteins from the total tissue lysate (30 μg) or mitochondrial fraction (15 μg) were separated on a 12% or 15% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was incubated for 2 h with blocking solution from Li-COR and then incubated overnight at 4 °C with primary antibody diluted in blocking solution supplemented with 0.1% (w/v) Tween 20. Primary antibodies used: UCP1 (1:1000, ab23841, Abcam), VDAC1 (1:500, sc-32063, Santa Cruz Biotechnology), COX IV (1:2 000, ab14744, Abcam), cytochrome c (1:1 000, 11940, Cell Signaling), Mito-

Profile Total OXPHOS Rodent WB antibody mixture (1:250, ab110413, Abcam), β-actin (1:10000, ab62676, Abcam). After four 10-min washes in PBS (Sigma) supplemented with 0.1% (w/v) Tween 20, the membrane was incubated with the corresponding secondary antibody diluted in blocking solution (1:10 000) supplemented with 0.1% (w/v) Tween 20 and 0.01% (w/v) SDS for 1 h at room temperature. The specific signals were detected by the Odyssey Infrared Imaging System (Li-COR, Lincoln, NE). The resulting images were quantified using Li-COR Image Studio (Version 2.0). Protein expression was normalized to the internal control β-actin.

Quantitative PCR—Total RNA was isolated from tissues using Tri-reagent according to the manufacturer’s protocol (Molecular Research Center, Cincinnati, OH). RNA was diluted to 20 ng/μl in nuclelease-free water (Sigma) and then diluted 1:10 (2 ng/μl) in nuclelease-free water just before use. 3 μl of diluted RNA (6 ng) was used in a 10-μl reaction with a single-reporter measurement. Pooled iBAT and ingWAT RNA from eight mice was used as a standard. Both standards and samples were run in duplicate using the 7900 Real-time PCR System (Applied Biosystems). Each transcript level was normalized to the level of cyclophilin in the sample. Primers of specific Taq-Man assay primers were obtained from Life Technologies, and their DNA sequences are available on request.

Statistical Analysis—Data are presented as the means ± S.D. Statistical analysis was performed by Student’s t test for single comparisons using GraphPad Prism 6 software.

RESULTS

The Brown Adipocyte Phenotype in ingWAT and iBAT in the Transition from the Cold Environment to Thermoneutrality

There are two essential characteristics of brown adipocytes that define their capacity for thermogenesis in a changing temperature environment. One is the relative content or stoichiometry of UCP1 to the respiratory components, and the second determines the dynamics of mitochondrial or brown adipocytes turnover. We have assumed that mitochondria turnover as a function of ambient temperature occurs in a largely fixed population of brown adipocytes in both iBAT and ingWAT (18). To elucidate the changes in the brown adipocyte phenotype as a consequence of changes in ambient temperature, we utilized the protocol described in Fig. 1A.

Histological images show the presence of only unilocular adipocytes characteristic for white fat after initial acclimation at 29 °C (Fig. 1B, 0 days 4 °C). But after 10 days of cold exposure, large regions of the tissue were populated by cells with the typical multilocular morphology of brown adipocytes. This multicellular morphology was substantially reduced after 14 days at 29 °C and completely lost after 21 days.

Synthesis and Degradation of Ucp1 mRNA and Protein in ingWAT and iBAT

Ucp1 in ingWAT—The main hallmark of the brown adipocyte phenotype within brown and white fat depots is the expression of UCP1. We determined the kinetics of induction and regression of the UCP1 and other mitochondrial components in brown adipocytes from iBAT and ingWAT during
Thermogenic Capacity of Brown Adipocytes

**Figure 1.** The induction of brite adipocytes in ingWAT during cold stimulation and their involution in thermoneutral conditions. **A**, scheme of the experimental design to assess brite adipocytes turnover. Young adult (9 weeks) female AXB8 mice were singly housed for 10 days at 4 °C, after 7 days of initial acclimation at 29 °C (control group), and subsequently reacclimated to 29 °C. iBAT and ingWAT were collected from 4 animals per group at the following time points: day 7 of initial acclimation (0d4 °C); days 1, 2, 3, 5, 7, and 10 at 4 °C; days 1, 3, 5, 7, 14, and 21 of reacclimation to 29 °C. **B**, hematoxylin and eosin-stained paraffin sections of ingWAT of cold-stressed and rewarmed mice.

We have shown above that in the transition from the thermoneutrality cold, the -fold induction of *Ucp1* mRNA was modest at both the mRNA (6-fold) and the protein (2-fold) levels, whereas the -fold induction in ingWAT was >100 for mRNA and protein. Given the pivotal role of the mitochondria in the function of brown adipocytes, the above results set up an interesting situation in which it is possible that the kinetics of induction support a simple economic model for cold adaptation in which mitochondria are degraded by mitophagy at thermoneutrality and replaced by mitochondrial biogenesis upon subsequent adrenergic stimulation in the cold, whereas other subcellular components of the brown adipocyte are preserved. Mechanisms for controlling expression of genes for *Ucp1* and components of mitochondrial respiration would be sufficient without whole cell turnover or renewal, which seems to be the prevailing model for brown adipocyte turnover.

**Mitochondrial Respiratory Proteins; Induction in the Cold (4 °C) and Degradation after Rewarming (29 °C)**

We have shown above that in the transition from the thermoneutrality cold, the half-life for UCP1 protein in iBAT after prolonged cold exposure is ~200 times longer than that for mRNA (20). Despite large differences in the kinetics of change in *Ucp1* mRNA compared with the protein during temperature transitions, the overall change of 100-fold was similar for mRNA and protein.

**Ucp1 in iBAT**—In contrast to ingWAT, the changes in UCP1 protein levels in iBAT during the transitions between 29 °C and 4 °C were small (Fig. 2, A and C). After 10 days of cold exposure, UCP1 increased ~2-fold and then returned to the pre-cold level after 14 days at 29 °C (Fig. 2C).

Under thermoneutral conditions, *Ucp1* mRNA levels in iBAT were comparable with induced levels in inguinal fat, and after 12 h of cold exposure a 6-fold increase of gene expression rapidly occurred and remained stable for the entire period in the cold (Fig. 2E). The mRNA levels in iBAT decreased abruptly after the end of cold stimulation, as in ingWAT, and reached pre-stimulation levels after 1 day at 29 °C, whereas protein levels did not differ significantly from those in cold conditions at this time point (Fig. 2, C and E). However, unlike iBAT, which in thermoneutral conditions sustains ~20% of induced *Ucp1* mRNA levels, in ingWAT the *Ucp1* mRNA levels were undetectable, constituting a fundamental difference in mechanisms regulating *Ucp1* mRNA levels in iBAT versus inguinal fat.

Thus, upon cold exposure, both iBAT and ingWAT responded with large induction of *Ucp1* gene expression; however, higher absolute levels were reached in iBAT. Although the -fold induction in the brite adipocytes was greater (100-fold), this is simply a consequence of the virtual absence of *Ucp1* mRNA expression in the ingWAT of mice at thermoneutrality. It is noteworthy that the onset and kinetics of the transcriptional response to changes in ambient temperature are similar for iBAT and ingWAT (Fig. 2G).

**Mitochondrial Respiratory Proteins; Induction in the Cold (4 °C) and Degradation after Rewarming (29 °C)**

We have shown above that in the transition from the thermoneutrality cold, the -fold induction of *Ucp1* in iBAT was modest at both the mRNA (6-fold) and the protein (2-fold) levels, whereas the -fold induction in ingWAT was >100 for mRNA and protein. Given the pivotal role of the mitochondria in the function of brown adipocytes, the above results set up an interesting situation in which it is possible that the kinetics of induction support a simple economic model for cold adaptation in which mitochondria are degraded by mitophagy at thermoneutrality and replaced by mitochondrial biogenesis upon subsequent adrenergic stimulation in the cold, whereas other subcellular components of the brown adipocyte are preserved. Mechanisms for controlling expression of genes for *Ucp1* and components of mitochondrial respiration would be sufficient without whole cell turnover or renewal, which seems to be the prevailing model for brown adipocyte turnover.

As was shown above, UCP1 turnover in the transition from cold to warm is very different in iBAT and ingWAT. The former showed minimal changes in protein content, whereas the latter showed major changes. Thus, to better understand the turnover states of brown adipocytes from ingWAT and iBAT of cold-exposed and rewarmed mice, we analyzed proteins of the mitochondrial membrane (19). Additionally, it has been reported that the half-life for UCP1 protein in iBAT after prolonged cold exposure is ~200 times longer than that for mRNA (20). Despite large differences in the kinetics of change in *Ucp1* mRNA compared with the protein during temperature transitions, the overall change of 100-fold was similar for mRNA and protein.
mitochondrial respiratory apparatus that varied in their location within the mitochondria. Additionally, we assessed whether patterns of protein expression were linked to submitochondrial locations. These include VDAC1 of the outer mitochondrial membrane, cytochrome c, which is loosely associated with the inner membrane, and COX IV localized in the inner mitochondrial membrane. Other representative subunits of the respiratory complexes (RC) including NDUFB8 (RC I), SDHB (RC II), UQCRC2 (RC III), COX IV (RC IV), MTCO1 (RC IV), and ATP5A (RC V) were selected because of the availability of antibodies.

As evident in Fig. 3, in ingWAT the expression patterns of VDAC1 and COX IV were very similar to UCP1 (Fig. 2B), although the -fold induction was much lower than UCP1 (−16 for VDAC1 and 14 for COX IV). An exception is cytochrome c, whose expression was stable in both cold and warm conditions. In iBAT, on the other hand, the expression profiles of these mitochondrial proteins were nearly constant (Fig. 3). An extension of this analysis to selected subunits of the mitochondrial respiratory complexes showed that in iBAT the patterns for UCP1 and selected mitochondrial proteins resembled each other (Fig. 4 and Fig. 5A). However, when a similar comparative analysis was done for inguinal fat, where the UCP1 varied ~100-fold during the transition from cold to warm, no other proteins matched UCP1. Even if you keep in mind that in inguinal fat non-brite cells with mitochondria are present, there are...
proteins in respiratory complexes, for example selected components of RC II and RC V, that do not significantly change levels of expression in going from the cold to the warm (Figs. 4 and 5). One possible explanation for our findings is the existence of different mechanisms of turnover for mitochondrial proteins associated with brown adipocytes (21–26), or as suggested by Klingenspor and co-workers (27) up-regulation during cold stimulation is not uniform for all subunits of each RC. This may also suggest critical and rate-limiting roles for specific subunits in the thermogenic process.

FIGURE 3. The kinetics of induction and regression of mitochondrial components in iBAT and ingWAT during reversible transitions from 29 to 4 °C. Above: protein expression analysis of selected mitochondrial proteins VDAC1, COX IV, and cytochrome c (CYT C) performed by using the whole tissue lysate (30 μg) of mice maintained at 4 or 29 °C for the different lengths of time. Proteins were detected on different membranes that were prepared in parallel from the same sample dilution (for iBAT and ingWAT separately). The protein loading control sample from only one of the membranes is shown. The number of mice was four for the time point. Representative images are shown. Below: quantitative analysis of Western blot results. Signals of the protein expression were normalized to that of β-actin and are shown as fold change relative to the control group. Each time point is the average value from four animals. The asterisks indicate statistically significant differences in protein expression between mice exposed to cold for 10 days and mice from selected time points in the experiment. Student’s unpaired t test: *, p < 0.05; **, p < 0.01.
Thermogenic Capacity of Brown Adipocytes

**iBAT**

| cold exposure (4°C) | reacclimation (29°C) |
|---------------------|----------------------|
| 0 0.5 1 2 3 5 7 10 1 5 7 14 21 |

**ingWAT**

| cold exposure (4°C) | reacclimation (29°C) |
|---------------------|----------------------|
| 0 0.5 1 2 3 5 7 10 1 5 7 14 21 |

- RC V-ATP5A
- RC III-UQCRNC2
- RC IV-MTCO1
- RC II-SDHB
- RC I-NDUFB8

**Graphs**

- Protein expression (fold change)

**RC I**

**RC II**

**RC III**

**RC IV**

**RC V**

**UCP1**

**Days**
mRNA expression level of the two subunits of ATP synthase, Atp5g1 and Atp5a1, in brown and white fat. We found that mRNA levels of \textit{Atp5g1} was 2.5-fold higher in ingWAT compared with iBAT in both cold and thermoneutrality conditions (Fig. 5B). Interestingly, mRNA levels of \textit{Atp5a1} are much higher in cold and warm conditions compared with \textit{Atp5g1} in both iBAT and ingWAT; however, they reached higher values in brown fat (6-fold higher expression in 29 °C and 2.5 higher expression in 4 °C compared with ingWAT) (Fig. 5B). These data are consistent with previous reports where almost all subunits of the ATP synthase are expressed at high mRNA levels (as are all other OXPHOS proteins) in brown fat mitochondria; the exception is the P1 isoform of subunit c (c-F0 subunit P1-isoform or ATP5G1). The level of ATP5G1 mRNA and protein is exceptionally low in brown adipocytes and is the rate-limiting component determining the low activity of the ATPase complex (17, 29, 30). Interestingly, in iBAT levels of ATP synthase subunits were unchanged after cold stimulation, whereas in ingWAT we noted an increase of expression of both Atp5a1 and Atp5g1 subunits (Fig. 5B).

In addition to overexpression of the UCP1 protein, the thermogenic recruitment process is accompanied by a major increase in mitochondrial biogenesis (31). The equivalent to the mitochondria number is the mtDNA content, and changes in the mtDNA reflect fluctuation in the mitochondria number in the cell. In our experiment mtDNA content increased 2-fold after 10 days of cold exposure in ingWAT and then decreased after 14 days of reacclimation to a pre-stimulation level (Fig. 5C). In iBAT, a slight increase in the amount of cytochrome \textit{b} DNA after 10 days at 4 °C was followed by a statistically insignificant decrease at day 14 in 29 °C (Fig. 5C). However, the total amount of mtDNA was 4-fold higher in iBAT compared with ingWAT after 10 days of cold exposure.

It is reasonable to assume that both mtDNA content and the expression of mitochondrial proteins in iBAT are stable and present at similar levels at 4 and 29 °C (Figs. 2, A and C, 3, and 4), whereas in ingWAT the expression of UCP1 and mitochondrial biogenesis is reflected by up-regulation of mitochondrial markers (VDAC1 and COX IV), as well as mtDNA content reveals dynamic changes during reversible transitions from

---

\textbf{FIGURE 4.} Expression of selected subunits of mitochondrial respiratory chain complexes in iBAT and ingWAT in cold and thermoneutrality conditions. \textit{Above:} protein levels of representative subunits of the mitochondrial respiratory complexes (RC) I-V, NDUFB8 (RC I), SDHB (RC II), UQRC2 (RC III), MTCO1 (RC IV), and ATP5A (RC V) were determined by using the whole tissue lysate (30 μg) of mice maintained at 4 or 29 °C for the different time lengths. Representative images are shown. Below: graphs present quantitative analysis of expression of subunits of the mitochondrial respiratory complexes and UCP1 protein in iBAT (filled bars) and ingWAT (open bars) as the average value from four individual animals. Signals of the protein expression are shown as -fold change relative to the control group (0 days). The asterisks indicate statistically significant differences in protein expression between mice exposed to cold for 10 days and mice from selected time point of the experiment; Student’s unpaired \textit{t} test: *, \textit{p} < 0.05; **, \textit{p} < 0.01; ***, \textit{p} < 0.001; ****, \textit{p} < 0.0001.

\textbf{FIGURE 5.} Effect of ambient temperature on UCP1, respiratory chain components and mitochondrial DNA turnover in iBAT and ingWAT. \textit{A,} line graphs of WB results (see Figs. 2A and 4) comparing the change in expression of UCP1 protein and selected subunits of mitochondrial respiratory chain during cold and warm acclimation. Signals of the protein expression are shown as a multiple of the levels in control group (-fold change). \textit{B,} expression of \textit{Atp5a1} and \textit{Atp5g1} mRNA. Data are the means ± S.E. from 4–6 mice in each group. \textit{C,} changes in mitochondrial DNA content. Mitochondrial DNA-encoded cytochrome \textit{b} measurement relative to the nuclear gene cyclophilin was analyzed for four mice in duplicate. Student’s unpaired \textit{t} test: *, \textit{p} < 0.05; **, \textit{p} < 0.01; ***, \textit{p} < 0.001. 

29 °C to 4 °C. Thus, a defining difference in brown adipocytes from WAT and iBAT concerns the susceptibility of mitochondrial turnover and stability for components of mitochondria and indicates that iBAT possesses comparatively similar thermogenic capacity in cold and thermoneutral environments, whereas in ingWAT thermogenic potential is temporary and occurs only at low ambient temperature.

**Thermogenic Potential of Brown Adipocytes in iBAT and ingWAT**

The relative thermogenic capacity of brown adipocytes in iBAT and those in white fat was estimated by determining the proportion of UCP1 protein expression to components of RC in mitochondria isolated from iBAT and ingWAT. This comparison was carried out according to a protocol aimed at achieving the maximal level of Ucp1 expression (Fig. 6A). The time of cold acclimation needed for the UCP1 protein and mRNA to reach their maximal level in ingWAT was 15 days (Fig. 6, B and C); therefore, this time point was chosen for further experiments.

Mitochondrial fractions were isolated from iBAT and ingWAT of two groups of mice, one exposed to 29 °C for 7 days (in text 0 days at 4 °C) and the other exposed to 4 °C for 15 days.
Expression levels of UCP1 and representative subunits of the mitochondrial RC I-V were determined using Western blot analysis (Fig. 7, A, B, and C). Expression of UCP1 in mitochondria isolated from ingWAT of cold-acclimated mice reached 60% that of the level in iBAT mitochondria (Fig. 7B).

To estimate the ratio of UCP1 to RC components in brite adipocytes, protein expression values from day 0 at 4 °C were subtracted from values obtained after 15 days of cold exposure. This value is variable because the reduction in these respiratory components were not constant, as evident in Figs. 4 and 5. It was assumed that at 29 °C brite adipocytes are not induced within ingWAT and have no signal corresponding to UCP1 (A). Thus, expression levels of RC components at 29 °C were treated as background originating from other non-brown adipocytes.

FIGURE 7. Thermogenic capacity of mitochondria from brown adipocytes from iBAT and ingWAT from cold-acclimated mice. A, levels of UCP1 and mitochondrial respiratory chain subunits in purified mitochondria isolated from iBAT and ingWAT from a pool of 4 mice acclimated to 29 °C for 7 days (0d4°C) or to 4 °C for 15 days. Levels of UCP1 (B) and mitochondrial respiratory chain proteins (C) in iBAT and ingWAT mitochondria isolated from mice acclimated to 29 °C for 7 days (0d4°C) or to 4 °C for 15 days. a.u., absorbance units. D, the ratio of UCP1 protein to respiratory complex subunits in mice acclimated to 4 °C. In ingWAT, respiratory complex protein levels from initial acclimation conditions were subtracted from values obtained after 15 days of cold exposure (asterisk). It was assumed that at 29 °C brite adipocytes are not induced within ingWAT and have no signal corresponding to UCP1 (A). Thus, expression levels of RC components at 29 °C were treated as background originating from other non-brown adipocytes.
as background originating from non-brown adipocytes (Fig. 7C). As evident in Fig. 7D, a comparison of the ratio of UCP1 to RC between brown and brite mitochondria from iBAT and ingWAT showed no significant differences for complexes I, II, and III, but for RC IV and V the ratios were 30 and 25% less in mitochondria isolated from ingWAT, respectively. Overall, the results indicate that brown adipocytes from iBAT and those from ingWAT have a similar thermogenic potential.

**DISCUSSION**

The morphological and molecular responses of white fat depots to a range of environmental conditions highlight its extraordinary plasticity (32). This primary response is the reversible interconversion of brown and white adipocytes in response to adrenergic signaling. At the cell level the interconversion is hypothesized to occur by two main mechanisms: proliferation of a stem cell/progenitor compartment for de novo production of brown adipocytes (33) or direct activation of genes of the brown adipocyte phenotype in fully differentiated white adipocytes (34). An alternative to the latter involves genome reprogramming by a trans-differentiation mechanism. Some reports support the direct, reversible conversion of white adipocytes into brown adipocytes during β-adrenergic stimulation (34–38). The discussion and indeed the research on the cellular mechanism on the conversion of the white fat phenotype to the brown has focused almost exclusively on the biosynthetic phase in which UCP1, mitochondrial biosynthesis, and other phenotypes of a brown adipocyte are acquired; however, equally important is the fate of the brown phenotype when adrenergic signaling ceases and the tissue returns, from all appearances, to the white fat state. Our comparison of the loss of the brown phenotype in iBAT and ingWAT indicates that a key distinguishing difference between these two sites of brown adipocytes expression is the character of the involution when animals in the cold are returned to thermoneutrality.

To understand the effects of kinetics of induction of Ucp1 mRNA and protein, we compared its expression in iBAT and ingWAT. Small variations in the level of UCP1 existed in iBAT; in general UCP1 was only induced by ~2-fold in any mouse that had been analyzed. The idea that a many-fold induction of UCP1 occurs in response to some inducer was not supported by the data. The reason for the ceiling on UCP1 induction is very simple: excessive UCP1 is toxic and will kill the brown adipocyte. For example, the aP2-Ucp1 transgenic mouse, as a hemizygote, is resistant to both dietary and genetic obesity because of increased thermogenesis that is not adrenergically regulated; however, in a mouse homozygous for aP2-Ucp1, the brown adipocytes are completely ablated, and the mouse is phenotypically indistinguishable from the Ucp1−/− mouse (39). This condition for expression will also apply to brown adipocytes in white fat deposits. Therefore, the limit for induction of UCP1 in ingWAT is determined by the same limiting capacity for UCP1 accumulation in a brown adipocyte. This suggests that modulating the number of brite cells programmed for a specific fat depot in a particular strain of mouse will be an effective strategy for modulating the content of brown fat thermogenesis.

Given that cell proliferation is not detected in inguinl white fat depots by incorporation of BrdU in mice exposed to the cold (18, 37) whereas a robust mitotic activity occurs in several types of non-brown adipocytes in iBAT but not in brown adipocytes themselves (18, 40), the proportion of non-adipocytes will not affect our quantitative estimates of brown adipocyte induction. The evidence strongly indicates that the major change occurring in ingWAT in response to β-adrenergic activity is the conversion of differentiated mature white adipocytes into brown adipocytes without significant changes in the proliferation of other cells in the tissue. Therefore, in ingWAT the 2-fold increase in mtDNA is associated with mitochondria biogenesis in white adipocytes differentiating into brown adipocytes. The 2-fold increase in mtDNA is compatible with the 100-fold increase in Ucp1 mRNA or protein, as the mtDNA resides in a compartment of the tissue, that is, the white adipocyte, that was previous silenced with respect to the brown phenotype. Such a ratio of induction will be observed whether white fat depots have a high or low number of white fat cells capable of differentiating into brown adipocytes. The number of cells capable of differentiating will determine the absolute level of UCP1. Because the brown adipocytes in iBAT are also not proliferating, the induction, which is ~2-fold for UCP1 but does not involve mitochondrial DNA proliferation, represents the adrenergically stimulated up-regulation of the resident brown adipocytes in iBAT.

To follow the mechanism of brown adipocyte induction during cold stimulation and then its involution in thermoneutrality, we performed a time course analysis of the expression of UCP1 protein, the crucial functional marker of cells capable of non-shivering thermogenesis, and selected mitochondrial proteins involved in electron transport activity. As shown in Fig. 2, B and D, UCP1 was undetected in ingWAT of mice adapted to 29 °C, but during cold exposure levels gradually increased 100-fold over 10 days. As shown in Fig. 7 this maximal level of UCP1 expression was similar to that accumulating in brown adipocytes of iBAT after 15 days in the cold. This temperature-sensitive induction was reversible, as UCP1 returned to prestimulation levels when the temperature was returned to 29 °C. In contrast to ingWAT, brown adipocyte content estimated by the level of UCP1 protein expression in iBAT were relatively stable. Similar stability was previously found in malnourished mice during the lactation period (41). Additionally, Figs. 3 and 4 show that the induction/involution of brite adipocytes and classical brown adipocytes resulted in parallel increases/decreases of UCP1 with the expression pattern of other mitochondrial proteins: VDAC1, COX IV, and mitochondrial respiratory complex I, II, III, and IV. However, the -fold change for these proteins in iBAT was generally much smaller than that observed in ingWAT. An exception is that some of the proteins, SDHB and ATP5A, remained relatively stable in both ingWAT and iBAT. If the goal of this study is to uncover mechanisms for essentially the complete loss of UCP1 in brite cells and retention of the complexes in iBAT, what is the meaning of the retention of individual components in ingWAT with normal white fat morphology? In a quantitative study of mitochondrial proteins in iBAT and WAT, Forner et al. (27) suggested the presence of heterogeneity of induction for each of the subunits of
Thermogenic Capacity of Brown Adipocytes

each respiratory complex and indicated specific, rate-limiting functions for particular subunits of respiratory chain in the process of thermogenesis. Also, the existence of different mechanisms of turnover for mitochondrial proteins may explain the retention of individual components of RC; e.g. the PINK1-Parkin pathway in Drosophila promotes selective turnover of membrane-bound RC subunits in a manner independent of conventional autophagy (23). Additionally, it has been proposed that the similarity of the turnover rate for UCP1 and other mitochondrial proteins is the result of a stimulatory effect of UCP1 on mitochondrial biogenesis (42) and parallel proteolytic rates, as was shown by Moazed and Desautels (43, 44).

In general, according to previous published data and the above results, iBAT maintains UCP1-dependent thermogenic capacity even in a thermoneutral environment, but the thermogenic capacity in ingWAT is transient, active at low ambient temperature or upon stimulation with β-adrenergic agonists but essentially non-existent above 23 °C.

Our experiment to estimate the thermogenic content of brown adipocytes from iBAT and WAT resembles a recent study by Nedergaard and co-workers (45), and both studies indicate that the thermogenic capacity is similar. Furthermore, the kinetic response of the Ucp1 gene transcription and the rate of degradation of the components determining the brown phenotype in the two cells types in the transition from the warm to the cold and vice versa is similar. The differences between brown and brite cells are found in the process of involution when mice at a fully cold-induced state are returned to thermoneutrality. At the mRNA level a mechanism exists that maintains the basal level of Ucp1 mRNA in iBAT to 20% of the fully induced level, whereas the Ucp1 mRNA in ingWAT is completely degraded. At the protein level, UCP1 is maintained at 50% of its maximally induced level in iBAT, whereas UCP1 in ingWAT is completely degraded. An important similarity between iBAT and WAT is the kinetic response of Ucp1 mRNA induction on cold exposure, which is virtually indistinguishable with 50% of maximal levels achieved in 12 h. These results complement accumulating evidence that classical brown and brite adipocytes are not the same cell phenotype either developmentally or in terms of selective biomarker profiles, although they share the same mechanism of induction (noradrenergic-mediated induction by cold), the same capacity for thermogenesis, and the primary purpose, which is protection of body temperature by non-shivering thermogenesis (7, 9, 28, 45). The thermogenic potential of a fully differentiated brown adipocyte in iBAT and WAT may be equivalent at the tissue level in an experiment with forced cold adaptation. However, because of differences in the stability of the brown phenotype in iBAT versus brite cells, in a normal living environment with constantly fluctuating ambient temperatures the thermogenic potential could be very different depending on which type of brown adipocyte resides in the tissue.

Acknowledgments—We thank Elzbieta Malinowska and Agnieszka Korytko for outstanding support in the management of the laboratory and mouse colony.

REFERENCES

1. Rothwell, N. J., and Stock, M. J. (1979) Regulation of energy balance in two models of reversible obesity in the rat. J. Comp. Physiol. Psychol. 93, 1024–1034
2. Cannon, B., and Nedergaard, J. (2004) Brown adipose tissue: function and physiological significance. Physiol. Rev. 84, 277–359
3. Wu, J., Cohen, P., and Spiegelman, B. M. (2013) Adaptive thermogenesis in adipocytes: Is beige the new brown? Genes Dev. 27, 234–250
4. Kozak, L. P., and Koza, R. A. (2010) The genetics of brown adipose tissue. Prog. Mol. Biol. Transl. Sci. 94, 75–123
5. Seale, P., Bjork, B., Yang, W., Kajimura, S., Chin, S., Kuang, S., Scime, A., Devarakonda, S., Conroe, H. M., Ejerud-Bromage, H., Tempst, P., Rudnicki, M. A., Beier, D. R., and Spiegelman, B. M. (2008) PRDM16 controls a brown fat/skeletal muscle switch. Nature 454, 961–967
6. Petrovic, N., Walden, T. B., Shabalina, I. G., Timmons, J. A., Cannon, B., and Nedergaard, J. (2010) Chronic peroxisome proliferator-activated receptor γ (PPARγ) activation of epidymal derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. J. Biol. Chem. 285, 7153–7164
7. Walden, T. B., Hansen, I. R., Timmons, J. A., Cannon, B., and Nedergaard, J. (2012) Recruited vs. nonrecruited molecular signatures of brown, “brite,” and white adipose tissues. Am. J. Physiol. Endocrinol. Metab. 302, E19–E31
8. Xue, B., Rim, J. S., Hogan, J. C., Couter, A. A., Koza, R. A., and Kozak, L. P. (2007) Genetic variability affects the development of brown adipocytes in white fat but not in interscapular brown fat. J. Lipid Res. 48, 41–51
9. Wu, J., Boström, P., Sparks, L. M., Ye, L., Choi, J. H., Giang, A. H., Khandekar, M., Virtanen, K. A., Nuutila, P., Schaart, G., Huang, K., Tu, H., van Marken Lichtenbelt, W. D., Hoeks, J., Enerbäck, S., Schrauwen, P., and Spiegelman, B. M. (2012) Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell 150, 366–376
10. Jimenez, M., Barbatelli, G., Allevi, R., Cinti, S., Seydoux, J., Giacobino, J. P., Mazzini, P., and Prentier, F. (2003) β3-Adrenoceptor knockout in C57BL/6 mice depressing the occurrence of brown adipocytes in white fat. Eur. J. Biochem. 270, 699–705
11. Vitali, A., Murano, I., Zingaretti, M. C., Frontini, A., Ricquier, D., and Cinti, S. (2012) The adipose organ of obesity-prone C57BL/6J mice is composed of mixed white and brown adipocytes. J. Lipid Res. 53, 619–629
12. Couter, A. A., Bearden, C. M., Liu, X., Koza, R. A., and Kozak, L. P. (2003) Dietary fat interacts with QTLs controlling induction of Pgc-1 alpha and Ucp1 during conversion of white to brown fat. Physiol. Genomics 14, 139–147
13. Guerra, C., Koza, R. A., Yamashita, H., Walsh, K., and Kozak, L. P. (1998) Emergence of brown adipocytes in white fat in mice is under genetic control: effects on body weight and adiposity. J. Clin. Invest. 102, 412–420
14. Kozak, R. A., Hohmann, S. M., Guerra, C., Rossmeisl, M., and Kozak, L. P. (2000) Synergistic gene interactions control the induction of the mitochondrial uncoupling protein (Ucp1) gene in white fat tissue. J. Biol. Chem. 275, 34486–34492
15. Xue, B., Couter, A., Rim, J. S., Koza, R. A., and Kozak, L. P. (2005) Transcriptional synergy and the regulation of Ucp1 during brown adipocyte induction in white fat depots. Mol. Cell Biol. 25, 8311–8322
16. Nedergaard, J., and Cannon, B. (2013) UCP1 mRNA does not produce heat. Biochim. Biophys. Acta 1831, 943–949
17. Houck, J., Andersson, U., Tvrälik, P., Nedergaard, J., and Cannon, B. (1995) The expression of subunit-c correlates with and thus may limit the biosynthesis of the mitochondrial F0-F1-ATPase in brown adipose tissue. J. Biol. Chem. 270, 7689–7694
18. Lee, Y. H., Pettokova, A. P., Konkar, A. A., and Granneman, J. G. (2015) Cellular origins of cold-induced brown adipocytes in adult mice. FASEB J. 29, 286–299
19. Puigserver, P., Herron, D., Gianotti, M., Palou, A., Cannon, B., and Nedergaard, J. (1999) Induction and degradation of the uncoupling protein thermogenin in brown adipocytes in vitro and in vivo: evidence for a rapidly degradable pool. Biochem. J. 284, 393–398
20. Jacobsson, A., Mühleisen, M., Cannon, B., and Nedergaard, J. (1994) The uncoupling protein thermogenin during acclimation: indications for pre-
adiogenesis during white adipose tissue development, expansion and regeneration. Nat. Med. 19, 1338–1344
34. Rosenwald, M., Perdikari, A., Rülicke, T., and Wolfrum, C. (2013) Bidirectional interconversion of brite and white adipocytes. Nat. Cell Biol. 15, 659–667
35. Cancello, R., Zingaretti, M. C., Sarzani, R., Ricquier, D., and Cinti, S. (1998) Leptin and UCP1 genes are reciprocally regulated in brown adipose tissue. Endocrinology 139, 4747–4750
36. Cousin, B., Bascands-Viguerie, N., Kassis, N., Nibbelink, M., Ambid, L., Casteilla, L., and Pénicaud, L. (1996) Cellular changes during cold acclimation in adipose tissues. J. Cell. Physiol. 167, 285–289
37. Himms-Hagen, J., Melnyk, A., Zingaretti, M. C., Ceresi, E., Barbatelli, G., and Cinti, S. (2000) Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. Am. J. Physiol. Cell Physiol. 279, C670–C681
38. Granneman, J. G., Li, P., Zhu, Z., and Lu, Y. (2005) Metabolic and cellular plasticity in white adipose tissue I: effects of β3-adrenergic receptor activation. Am. J. Physiol. Endocrinol. Metab. 289, E608–E616
39. Stefl, B., Janovská, A., Hodný, Z., Rossmeisl, M., Horáková, M., Syrový, L., Běnová, J., Bendlová, B., and Kopecký, J. (1998) Brown fat is essential for cold-induced thermogenesis but not for obesity resistance in aP2-Ucp1 mice. Am. J. Physiol. 274, E527–E533
40. Bukowiecki, L., Collet, A. J., Follea, N., Guay, G., and Jahjah, L. (1982) Brown adipose tissue hyperplasia: a fundamental mechanism of adaptation to cold and hyperphagia. Am. J. Physiol. 242, E353–E359
41. Kozak, L. P., Koza, R. A., Annuciado-Koza, R., Mendoza, T., and Newman, S. (2012) Inherent plasticity of brown adiogenesis in white fat of mice allows for recovery from effects of post-natal malnutrition. PloS ONE 7, e30392
42. Rossmeisl, M., Barbatelli, G., Flachs, P., Brauner, P., Zingaretti, M. C., Marelli, M., Janovská, P., Horáková, M., Syrový, L., Cinti, S., and Kopecký, J. (2002) Expression of the uncoupling protein 1 from the aP2 gene promoter stimulates mitochondrial biogenesis in unilocular adipocytes in vivo. Eur. J. Biochem. 269, 19–28
43. Moazed, B., and Desautels, M. (2002) Differentiation-dependent expression of cathepsin D and importance of lysosomal proteolysis in the degradation of UCP1 in brown adipocytes. Can. J. Physiol. Pharmacol. 80, 515–525
44. Moazed, B., and Desautels, M. (2002) Control of proteolysis by norepinephrine and insulin in brown adipocytes: role of ATP, phosphatidylinositols 3-kinase, and p70 S6K. Can. J. Physiol. Pharmacol. 80, 541–552
45. Shabalina, I. G., Petrovic, N., de Jong, J. M., Kalinovich, A. V., Cannon, B., and Nedergaard, J. (2013) UCP1 in brite/beige adipose tissue mitochondria is functionally thermogenic. Cell Rep. 5, 1196–1203

Thermogenic Capacity of Brown Adipocytes