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The expression of UCP3 directly correlates to UCP1 abundance in brown adipose tissue

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Abbreviations:

BAT, brown adipose tissue; He, heart; GMsc, gastrocnemius muscle; mUCP3, recombinant uncoupling protein 3; RT, room temperature; ScM, scapulae muscle; SkM, skeletal muscle; Sp, spleen; Th, thymus; WB, Western blot

Abstract

UCP1 and UCP3 are members of the uncoupling protein (UCP) subfamily and are localized in the inner mitochondrial membrane. Whereas UCP1's central role in non-shivering thermogenesis is acknowledged, the function and even tissue expression pattern of UCP3 are still under dispute. Because UCP3 properties regarding transport of protons are qualitatively identical to those of UCP1, its expression in brown adipose tissue (BAT) alongside UCP1 requires justification. In this work, we tested whether any correlation exists between the expression of UCP1 and UCP3 in BAT by quantification of protein amounts in mouse tissues at physiological conditions, in cold-acclimated and UCP1 knockout mice. Quantification using recombinant UCP3 revealed that the UCP3 amount in BAT (0.51 ng/(µg total tissue protein)) was nearly one order of magnitude higher than that in muscles and heart. Cold-acclimated mice showed an approximate three-fold increase in UCP3 abundance in BAT in comparison to mice in thermoneutral conditions. Surprisingly, we found a significant decrease of UCP3 in BAT of UCP1 knockout mice, whereas the protein amount in skeletal and heart muscles remained constant. UCP3 abundance decreased even more in cold-acclimated UCP1 knockout mice. Protein quantification in UCP3 knockout mice revealed no compensatory increase in UCP1 or UCP2 expression. Our results do not support the participation of UCP3 in thermogenesis in the absence of UCP1 in BAT, but clearly demonstrate the correlation in abundance between both proteins. The latter is important for understanding UCP3`s function in BAT.

Introduction

UCP1 and UCP3, proteins of the mitochondrial uncoupling protein subfamily (UCP), are implicated in the pathophysiology of different diseases such as obesity, diabetes mellitus, ischemia, cancer, etc. (1-3). However, the underlying molecular mechanisms and even the protein distribution among tissues have been controversially discussed for nearly two decades.

UCP3, first described in muscles (4), has 57% and 55% homology to UCP1 in humans and mice, respectively. Both UCP1 and UCP3 were shown to transport protons (5-7). However, whereas the role of UCP1 in non-shivering thermogenesis is accepted, the function of UCP3 is still under dispute (8-10). Alternatively, UCP3 was proposed to transport fatty acids, lipid peroxides and pyruvate (11-14). Results from experiments using knockout or overexpression of UCP3 in murine skeletal muscles imply the protective role of UCP3 against triglyceride accumulation (15;16).

Although the expression of UCP3 at the protein level was reported for muscle (17), heart (18), brown adipose tissue (19), white adipose tissue (20), spleen and thymus (21), the investigation of its function has mostly been focused on how it works in muscles. The generation of UCP1 and UCP3 knockout mice reveal that such mice have no conspicuous phenotype under physiological conditions (22;23). The cold adaptation of a UCP1 knockout mouse confirmed the function of UCP1 as a mediator of non-shivering thermogenesis (24;25). Because both UCP1 and UCP3 are localized in BAT, it is possible that the function of one protein can be taken over by the other (sister) uncoupling protein. One of the few existing comparative investigations of UCP1 and UCP3 describes the mRNA interdependence of UCP1 in BAT with UCP3 in skeletal muscles (26). However, in contrast to UCP1, UCP3 is characterized by a rapid turnover (27) which makes protein expression analysis so important in the investigation of UCP3 function and regulation. It was reported that UCP3 abundance increases in skeletal muscle of cold-acclimated UCP1$^{-/-}$ mice, however, no evidence was found to indicate it takes over the UCP1 thermogenic function (28).

The understanding of UCP3´s expression pattern under different physiological and challenging (cold acclimation) conditions will provide an important indication for revealing its function. Therefore, the goals of the present work were (i) to quantify the expression of UCP3 in mouse tissues using the UCP3 recombinant protein and a novel polyclonal antibody; (ii) to evaluate whether and in which tissues UCP1 and UCP3 may substitute for each other by compensatory increases in protein abundance, using the model of UCP1 and UCP3 knockout mice and (iii) to verify whether there is any interaction between UCP1 and UCP3 at the protein level in general.

2. Materials and methods

2.1 Chemicals

All of the chemicals we used, except for those mentioned below, were acquired by Roche (Austria), Sigma Aldrich (Austria), Biorad (Austria), Amersham Biosciences, GE Healthcare (Austria).

2.2 Recombinant proteins

Recombinant proteins, murine UCP1 (mUCP1), human UCP2 (hUCP2), mUCP2, mUCP3, mUCP4-eGFP and mUCP5, were used for antibody validation and for quantification as a standard in Western blot analysis. Recombinant production of mUCP1, hUCP2, mUCP4-eGFP and mUCP5 has been previously described (29;30). Production and reconstitution of mUCP2 and mUCP3 were performed in a similar fashion. In brief, the open reading frames of mUCP2 and mUCP3 were obtained as full length cDNA clones IRAVp968G0921D and IRCKp5014J1815Q (BioCat, Germany). Mouse *ucp2* was inserted in the NdeI and EcoRI sites, mouse *ucp3* - in the NheI and a BamHI sites of pET24a+ (Novagen, Germany), respectively. For protein expression, pET24a+ containing *mucp2* or *mucp3* were transfected into the *E. coli* expression strain Rosetta (DE3) (Novagen, Germany). Bacteria were grown to OD 600 0.3 – 0.5 before protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After incubation for an additional 2.5 hours, bacteria were harvested by centrifugation. A suspended bacteria pellet was lysed using a French press (Constant Systems Limited, UK). Inclusion bodies were collected by centrifugation at 14.000g. Approximately 3 mg of mUCP2 or mUCP3 were solubilized from inclusion bodies using 2% sarcosyl and 1 mM DTT. 100 mg *E. coli* polar lipid (Avanti polar lipids, Alabaster, USA), 300 µg Triton X-114, 75 µg octyl-polyoxyethylene and 2 mM GTP were added to the solubilized mUCP2 or mUCP3. Sarcosyl and GTP were removed by dialysis, and the buffer was thereby changed to the assay buffer (50 mM $NaSO_4$, 10 mM Tris, 10 mM MES and 0.6 mM EGTA, pH 7.34). The dialysate was passed through a hydroxyapatite column (Bio-Rad, Germany) to remove decomposed proteins. Non-ionic detergents were eliminated using Bio Beads (Bio-Rad, Germany). Proteoliposomes containing mUCP2 or mUCP3 were stored at -80°C.

2.3 Animals

UCP1$^{-/-}$ (24), UCP2$^{-/-}$ (31), UCP3$^{-/-}$ (17) and their wild type (wt) controls used in this study were backcrossed for 10 generations on the C57BL/6 background. Alternatively, three months old wt C57BL/6 mice were used for the experiments on isolated mitochondria. The animals were kept in a 12 h:12 h light-dark cycle, were acclimated to 21-23°C (unless otherwise stated) for at least 4 weeks and had unlimited access to food (R70 Standard Diet, Lactamin) and tap water. Mice were single caged, cages were enriched with wooden chips, cartoon tube and paper for mice welfare. All experiments were approved by the Animal Ethics Committee of the North Stockholm region, Sweden.

2.4 Tissue Samples Preparation

Animals were anesthetized with a mixture of 79% CO_2 and 21% O_2 for 1 minute and then decapitated. The extracted tissues were immediately shock-frozen in liquid nitrogen for further investigation. We applied two homogenisation protocols for different tissues (30;32). To homogenize brown (BAT) and inguinal white (WAT, (20)) adipose tissues, we used a Potter homogenizer with a Teflon pestle. BAT and WAT were homogenized in RIPA buffer supplemented with a protease inhibitor (Complete mini). After centrifugation (14000g, 15 min, at 4°C), the supernatant was collected with a syringe, aliquoted and stored at -80°C. Thymus, spleen, brain and lungs were crushed using a mixer mill (MM200, Retsch, Germany) in lysis buffer (50 mM Tris, 150 mM NaCl, 1% sodium deoxycholate, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, pH 7.4) with a protease inhibitors cocktail and then sonicated. Heart (He), skeletal muscles (SkM) and stomach were first trimmed into smaller pieces with scissors and then treated as described above. After 30 minutes of incubation on ice, the lysates were centrifuged 10 minutes at 2500g. The supernatants were collected, centrifuged again under same conditions, aliquoted and stored at -80°C. The total protein concentration was determined with a Pierce BCA Protein Assay Kit (Thermo Scientific).

2.5 Mitochondrial protein isolation

BAT, WAT, He and SkM mitochondria were used for the UCP3 antibody validation and UCP3 quantification. Extraction of BAT, WAT, He and SkM (skeletal muscles: scapulae muscle, ScM and gastrocnemius muscle, GMsc) and mitochondria isolation were performed according to two different protocols (28) and (30) with a modification as proposed in (33).

2.6 Western blot analysis

Western blot (WB) analysis was performed as described previously (34). We modified the described protocol by using PVDF membranes (Amersham Hybond-P PVDF Transfer Membrane GE Healthcare). Probes were incubated at 4°C overnight (or at least for 8 hours) with the primary antibody diluted in 2% BSA solution (bovine serum albumin, Sigma Aldrich, Austria) and for 1 hour at RT with the secondary antibody diluted in 2.5% milk solution. Detection was performed by luminescence reaction using a secondary antibody against rabbit antibodies linked with horseradish peroxidase (GE Healthcare, Austria) and ECL Western Blotting reagent (GE Healthcare, Austria) accomplished with a ChemiDoc-It® 600 Imaging System (UVP, UK). The Launch Vision Works LS software (UVP, UK) was used for quantification. To ensure an accurate comparison of UCP1 and UCP3 amounts in different tissues or under different conditions, we methodically controlled the exposure time to avoid signal saturation using the same software.

To control protein loading, the membranes were stripped with a low pH strip solution (100 mM sodium citrate at pH 2.2) for at least 1 minute, washed, blocked for 30 minutes in block solution and incubated again with antibodies against SDHA (succinate dehydrogenase complex subunit A, Abcam ab14715; dilution 1:5000), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase, Sigma-Aldrich, G8795; dilution 1:10000) and NDUFA9 (complex I, Invitrogen, 459100; dilution 1:3000) diluted in 2% BSA solution for at least 1 hour at RT, and subsequently incubated with horseradish peroxidase-linked anti-mouse IgG (GE Healthcare, NA931).

2.7 Statistics

In each experiment 3-5 mice were used per group. All data are presented as mean values ± standard deviation (SD). Mean value for each mouse was build as a mean value of 2-3 Western Blots at the same conditions. Statistical analyses for the comparison of two mice groups were performed using Student's t test. By more than two mice groups one-way ANOVA test was used. *, **, *** indicate significant difference of $p<0.001$, $p<0.004$ and $p<0.012$ respectively. The analysis was carried out using Sigma Plot 12.5 software.

3. Results

3.1 UCP3 antibody generation and validation

The quality of antibodies is crucial for precise assessment of UCP tissue distribution. Because the commercially available antibodies against mUCP3 were not adequately specific enough for our research purposes, we generated a new polyclonal antibody against the peptide sequence located in the third matrix loop of the protein. We used tissue samples from UCP3 knockout mice as a negative and recombinant UCP3 as a positive control to verify the specificity of the novel antibody in WB experiments. Recombinant mUCP1, mUCP2, mUCP4-eGFP, mUCP5 and mUCP3 (30;35) were used to exclude a cross-reactivity with other UCP family members. Figure S1 (Supplementary Materials) demonstrates that the anti-mUCP3 antibody we produced recognized recombinant mUCP3 and UCP3 in skeletal muscle (SkM, Figure S1, A) of wt mice and showed no cross-reactivity with other recombinant UCPs (Figure S1, B).

3.2 UCP3 distribution in murine tissues at protein level

First, we compared the protein distribution in tissues of wt and UCP3 knockout mice under physiological conditions to evaluate the controversial reports about UCP3 tissue localization described in Introduction. Figure 1, A demonstrates that UCP3 is expressed in brown adipose tissue (BAT), inguinal white adipose tissue (WAT), skeletal muscle (SkM) and heart (He) of wt mice, but not in the knockout controls. We did not detect UCP3 in the liver (Li), kidney (Ki), brain (B), testis (Tes), thymus (Th), spleen (Sp), lungs (Lu) and stomach (St) (Figure 1, B-C). This expression pattern generally confirms the previous reports (23;36). Interestingly, UCP3 expression seems to be different in rats compared with mice; UCP3 was described in the murine thymus and spleen (although in very low abundance), but no UCP3 was found in the rat heart (21;37).

The data imply that UCP3 abundance in BAT is distinctly higher than in SkM. To verify that this difference is not related to the mitochondria amount, we repeated the experiment using isolated mitochondria from heart, muscles and BAT. Figure 1, D shows that the UCP3 amount in mitochondria isolated from BAT is indeed approximately two times higher than in skeletal muscles.

3.3 Quantification of UCP3 expression at physiological conditions

To quantify the differences between the expression of UCP3 in BAT, heart and muscles, we used the recombinant mouse protein (mUCP3). Increasing concentrations of mUCP3 and the investigated tissues (20 and 50 µg of total protein) were gradually loaded onto a WB gel. We plotted the values of WB density against known mUCP3 concentration (Figure 2, A-D) and calculated the UCP3 content per 1 µg total cellular protein (Fig. 2, E) to compare UCP3 abundance among different investigated tissues. The UCP3 amount in BAT (0.51 ± 0.11) ng/(µg total protein) was nearly one order of magnitude higher than that in gastrocnemius muscle (0.058 ± 0.024) ng/(µg total protein), scapulae muscle (0.038 ± 0.028) ng/(µg total protein), and heart (0.093 ± 0.02) ng/(µg total protein). We performed the quantification in isolated mitochondria in a similar manner (Figure 2, F). The results correspond with those obtained with tissue. No difference was observed between two diverse types of skeletal muscle tissue, which contradicts the previously reported results (33).

3.4 Quantification of UCP3 expression in mice housed at different temperatures

Next, we evaluated whether UCP3 expression is temperature-sensitive akin to UCP1. For this, we measured UCP3 in BAT, ScM, GMsc and He tissue probes taken from mice housed at 4°C (cold acclimatization), 21°C (standard housing) and 30°C (thermoneutral control). Indeed, Figure 3, A shows that cold acclimatization of mice led to a three-fold increase of UCP3 abundance in BAT (0.61 ng/(µg total protein) at 4°C vs. 0.22 ng/(µg total protein) at 30°C). To verify that the observed UCP3 increase was not due to an increase in mitochondria number, we performed UCP3 quantification using isolated mitochondria. Figure 3, B confirms that UCP3 abundance is temperature-sensitive and is up-regulated at lower temperatures. Surprisingly, no change in UCP3 abundance was measured in skeletal muscles and heart under similar temperature conditions (Figure 3, C-E).

3.5 Uncoupling proteins expression patterns in UCP1 or UCP3 - ablated mice

To evaluate whether the lack of UCP1 can be substituted by the up-regulation of UCP3 or *vice versa* at physiological conditions, we determined the UCP3 expression in several tissues of the $UCP1^{-/-}$ mice (Figure 4) and UCP1 expression in $UCP3^{-/-}$ mice (Figure 5). We detected a significantly lower UCP3 expression in BAT of $UCP1^{-/-}$ compared to wt mice, whereas heart and both types of skeletal muscles showed similar UCP3 abundance (Figure 4, A). To test whether the temperature decrease would stimulate UCP3 expression, we compared $UCP1^{-/-}$ mice at 4°C

and 21°C (Fig. 4, B). Surprisingly, UCP1$^{-/-}$ mice acclimated at 4°C showed the lowest UCP3 amount. Because of high homology between UCP2 and UCP3 (~75%), we analysed whether UCP2 is regulated in UCP1$^{-/-}$ mice. The results revealed that UCP2 is not expressed in brown adipose tissue as a compensation for the lack of UCP1, and its abundance in tissues normally expressing UCP2 (spleen and thymus (35)) does not change (Figure S2, A-B, Supplementary Materials).

To test whether the UCP1 expression level is also influenced by the absence of UCP3, we measured it in UCP3$^{-/-}$ mice. UCP1 was neither expressed in SkM nor He (Figure 5, A), and was not up-regulated in BAT (Figure 5, B). Similarly, no compensatory expression of homologous UCP2 was detected in tissues where UCP3 (BAT, SkM or He, Figure S2, C) or UCP2 (Sp and Th, Figure S2, D) are normally expressed.

3.6 Uncoupling proteins expression patterns in UCP2 - ablated mice

Finally, we analysed four UCP2$^{-/-}$ mice to test for the compensatory up-regulation of UCP3 or UCP1. We did not find expression of UCP1 or UCP3 (data not shown) in tissues where UCP2 is normally expressed (35). BAT in UCP2$^{-/-}$ mice had a similar amount of UCP1 and UCP3 compared to the wt controls (Figure S3, A-B). No compensatory expression of UCP3 was detected in skeletal muscle or heart (Figure S3, B).

4. Discussion

Although one of the first reports about UCP3 described its expression in muscles and BAT (36) more recent morphological and functional studies generally refer to UCP3's predominance in skeletal muscles. The lack of a commercially available, well characterized antibody is still the main obstacle for a reliable comparison of UCP3 abundance in different tissues. For this study we first evaluated a self-designed antibody using positive (recombinant protein) and negative (knockout) controls. The quantitative analysis of UCP3 abundance in skeletal muscles, heart, and brown adipose tissue of mice revealed that BAT has the highest UCP3 amount among the investigated tissues even at physiological conditions. This difference was even more obvious in tissues of cold-acclimated mice. We verified this finding by testing isolated mitochondria to exclude artefacts caused by different amounts of mitochondria in various organs and the adaptive mitochondriogenesis described for cold acclimation (38). The different expression of rat UCP3

mRNA in BAT in comparison to skeletal muscle after exposure to cold was reported earlier (39). The higher amounts of expressed protein in BAT and its expression alongside UCP1 may reflect a specific role of UCP3 in this tissue compared to muscles and heart.

Quantitative analysis using recombinant proteins further demonstrated that the abundances of UCP3 and UCP1 in BAT are directly correlated. The functional links between UCP1 and UCP3 were analysed in mice at physiological conditions, in cold-acclimated, $UCP1^{-/-}$ and $UCP3^{-/-}$ mice. Similar to UCP1, UCP3 in BAT was regulated by temperature showing three times higher expression at 4°C than at 30°C (thermoneutrality). At first glance, these results would suggest a thermogenic role of UCP3 in BAT as proposed earlier (39). However, we did not find any compensatory expression of UCP3 in tissues of $UCP1^{-/-}$ mice, in which the ablated UCP1 is normally present. We even detected a significant decrease of UCP3 in BAT of $UCP1^{-/-}$ animals, in contrast to previously reported higher expression of UCP3 in skeletal muscles of cold-acclimated UCP1 knockout mice (28). This fact rather indicates that UCP3 is not able to take over the thermogenic function of UCP1 in BAT, again supporting the hypothesis about an organ-specific function for UCP3. Moreover, UCP3 abundance in BAT is still approximately 400-fold lower, when compared to UCP1 amounts (35).

The various expression of UCP3 in BAT and muscles may be explained by the recently described differential gene regulation in both tissues (40). In BAT, the UCP3 transcription is stimulated by an additional intronic enhancer with SP1/3 and PPARγ as core factors for *ucp3* gene expression. Also a posttranslational regulation - phosphorylation (41) or glutathionylation (42) - was proposed to influence the uncoupling function of UCP3 and may differ in BAT and skeletal muscles.

We can only speculate about the molecular function of UCP3 and its interconnection with UCP1, as is similar in other groups in this research area to date. The direct correlation between UCP1 and UCP3 amounts in BAT could most likely be explained by the specific UCP3 transport function (43)), coherent with the proton transport function of highly abundant UCP1. This idea is supported by other groups that have previously reported on UCP3 exporting fatty acids from mitochondria predominantly during fatty acid oxidation (22). Bouillaud et al. (44) suggested a UCP3 function in pyruvate and glutamine metabolism as a passive pyruvate transporter. This suggestion would fit to the putative involvement of UCP3 in the regulation of brown fat cell metabolism similar to the function proposed for the highly homologous UCP2 (73%) in rapidly proliferating cells (34;35).

In conclusion, we show that (i) UCP3 is highly abundant in BAT and the sensitivity of the protein expression to temperature is similar to that of UCP1; (ii) UCP3 expression is significantly decreased when UCP1 is knocked out. Further investigation of the molecular role of UCP3 in BAT is an important task for the future, and may provide new insights into its function.

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Figure Description

Figure 1. UCP3 expression patterns in murine tissues under physiological conditions. (A) UCP3 was detected in skeletal muscle (SkM), heart (He), brown adipose tissue (BAT) and white adipose tissue (WAT) of wild type (wt) mice. Corresponding tissues from UCP3$^{-/-}$ mice were used as a negative control. (B-C) Kidney (Ki), liver (Li), brain (B), testes (Tes), thymus (Th), spleen (Sp), lung (Lu) and stomach (St) show no detectable UCP3 expression. 25 μg of total protein from the tissue were loaded per lane. Five mice per group were tested. (D) UCP3 expression in isolated mitochondria. 20 μg isolated mitochondria from He, SkM and 10 μg isolated mitochondria from BAT were loaded per lane. Recombinant mouse UCP3 (mUCP3, 5 ng) was used as a positive control. Antibodies against GAPDH, SDHA and NDUFA9 were used as a control for protein loading and mitochondria amount. Six mice were tested.

Figure 2. Protein quantification in tissues expressing UCP3. (A-D) Representative WBs for UCP3 quantification in BAT (A), He (B), ScM (C) and GMsc (D) in wt mice under physiological conditions. 20 μg and 50 μg of total protein from the investigated tissue (red circles) and different concentrations of recombinant UCP3 (mUCP3) were loaded for quantification. (E-F) Comparison of UCP3 amounts in different tissues (n=4, E) and in isolated mitochondria (n=7) of wt mice (F).

Figure 3. Quantification of UCP3 expression at different temperatures. (A) UCP3 amount in tissue lysates from brown adipose tissue, (C) heart, (D-E) skeletal muscles (ScM, D; GMsc, E) and (B) mitochondria isolated from BAT. Wild type mice were housed at 30° C, 21° C and 4° C. 20 μg of total protein from tissue or 10 μg isolated mitochondria were loaded per lane. The relative UCP3 amount (r.u.) is a ratio between the band intensity of standard (mUCP3, 5 ng) and sample. Five mice were tested at 30°C and 4°C and four mice at 21°C.

Figure 4. Representative Western blots and quantification showing UCP3 expression in UCP1$^{-/-}$ mice. (A) UCP3 expression in brown adipose tissue, skeletal muscles (ScM, GMsc) and heart of UCP1$^{-/-}$ mice compared to their wild type controls (wt). Data are presented as a mean values from three mice ± SD. (B) UCP3 expression in BAT of UCP1$^{-/-}$ mice housed at different temperatures. The relative amount (r.u.) of UCP3 is a ratio between the band intensity of standard (mUCP3, 5 ng) and sample. 25 μg of total protein from tissues were loaded per lane. The number of tested mice was n=5 at 4°C and n=4 at 21°C.

Figure 5. Representative Western blots and quantification showing UCP1 expression in UCP3$^{-/-}$ mice. (A) No compensatory expression of UCP1 in skeletal muscles, heart and (B) BAT. Data are presented as a mean values from five mice ± SD. 20 μg of total protein from tissues of UCP3$^{-/-}$ or wt mice were loaded per lane. The relative protein amounts of UCP1 were calculated as a ratio between line intensity of standard (mUCP1, 250 ng) and sample.

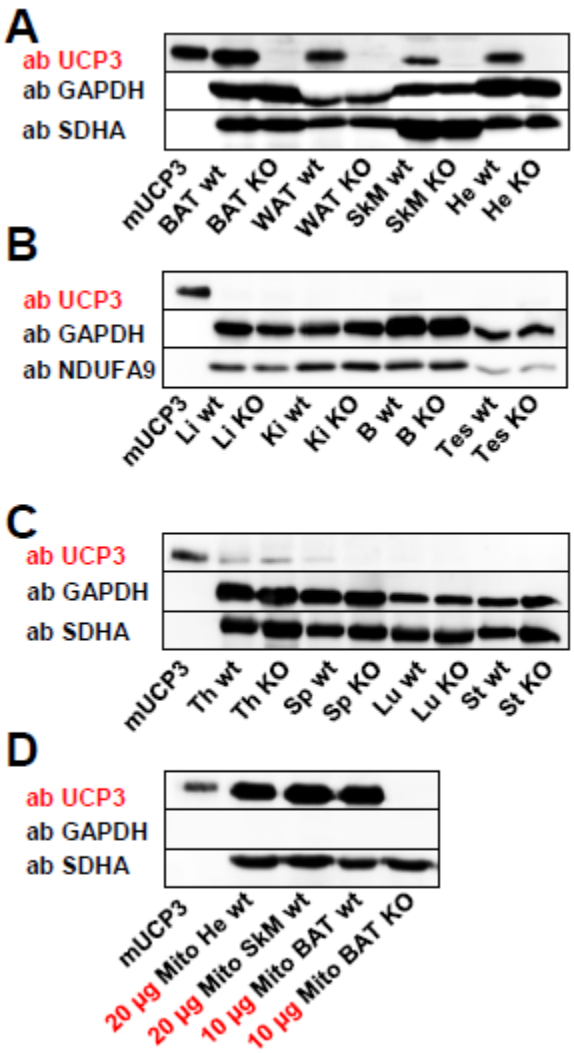

Figure 1

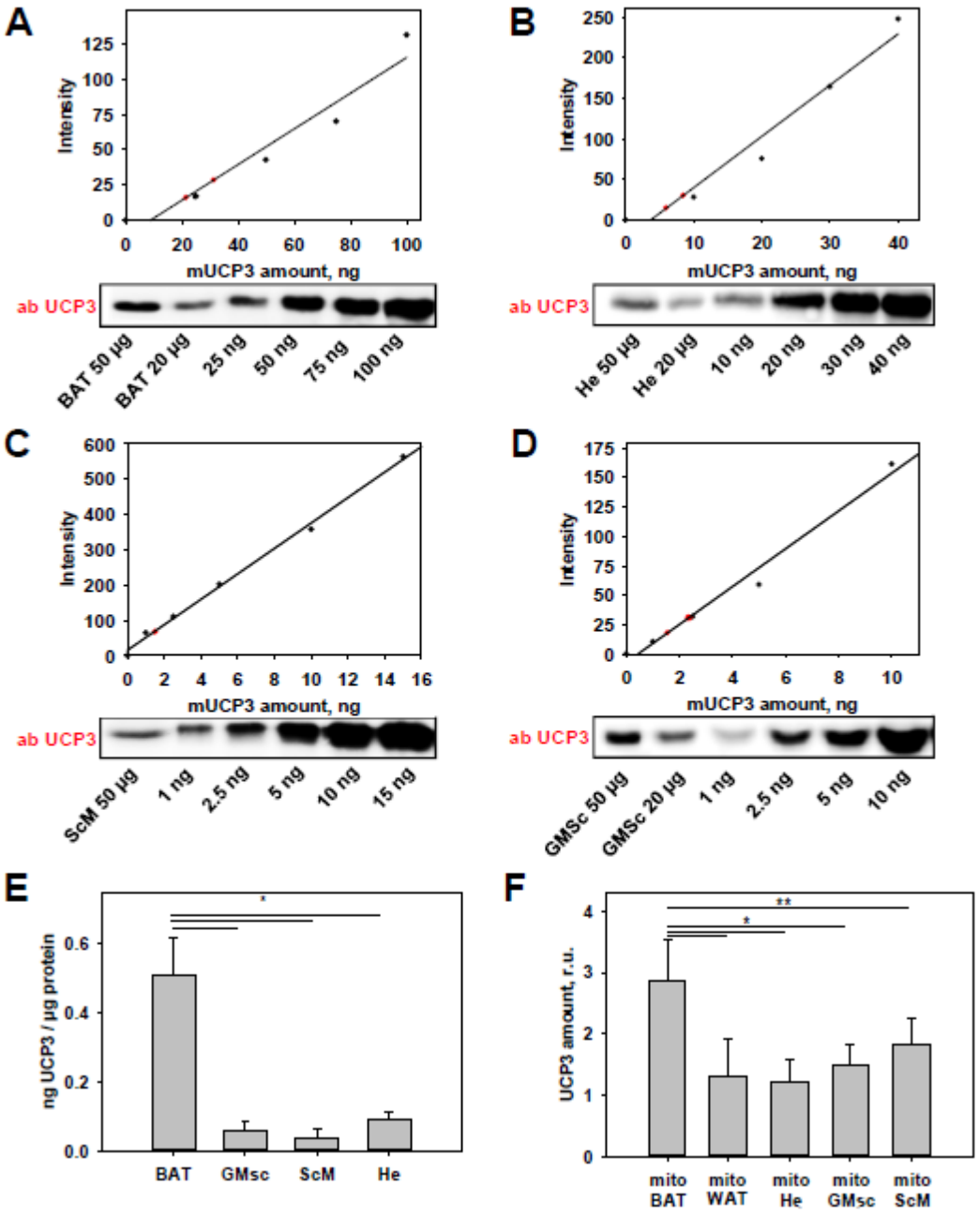

Figure 2

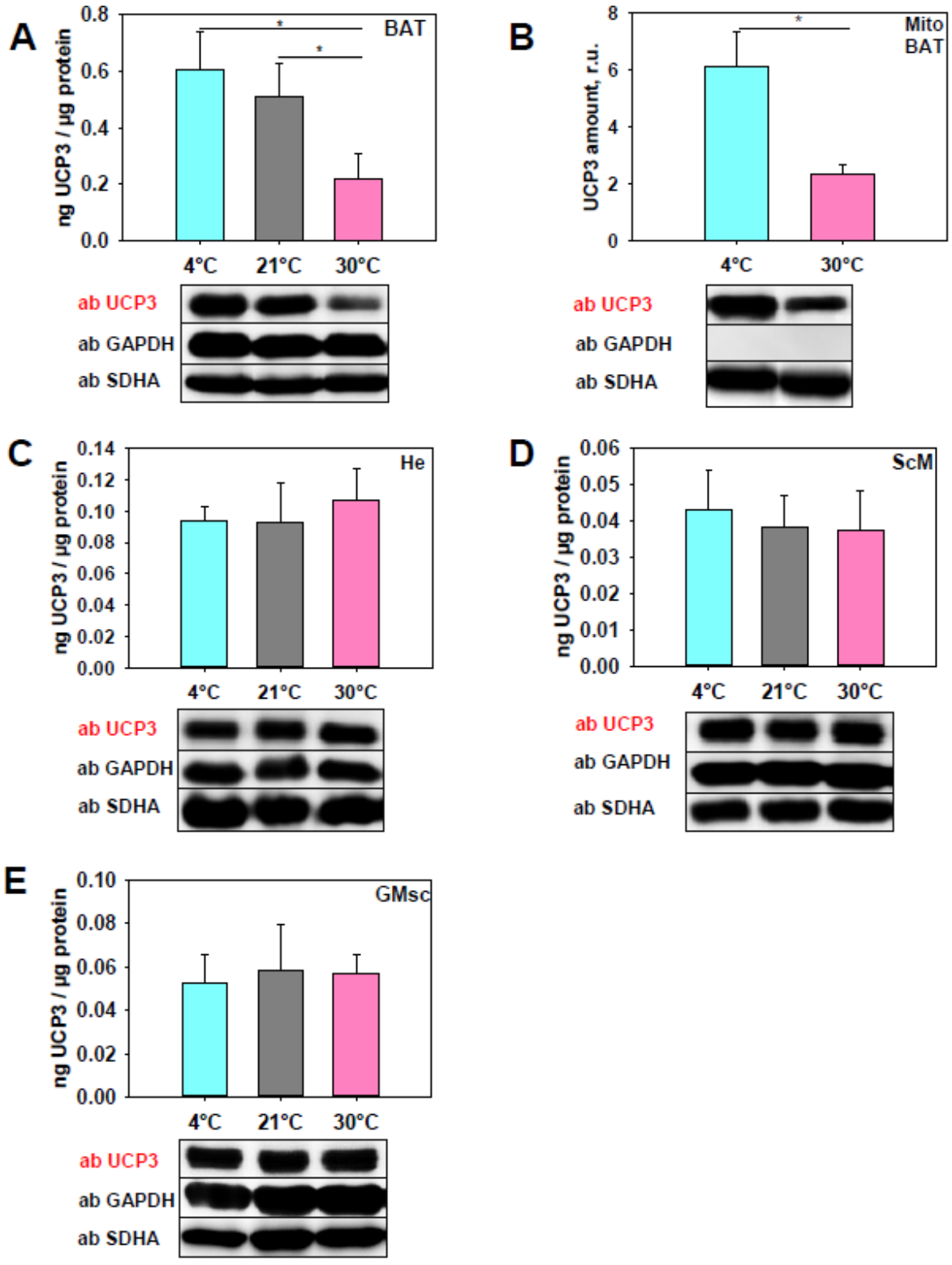

Figure 3

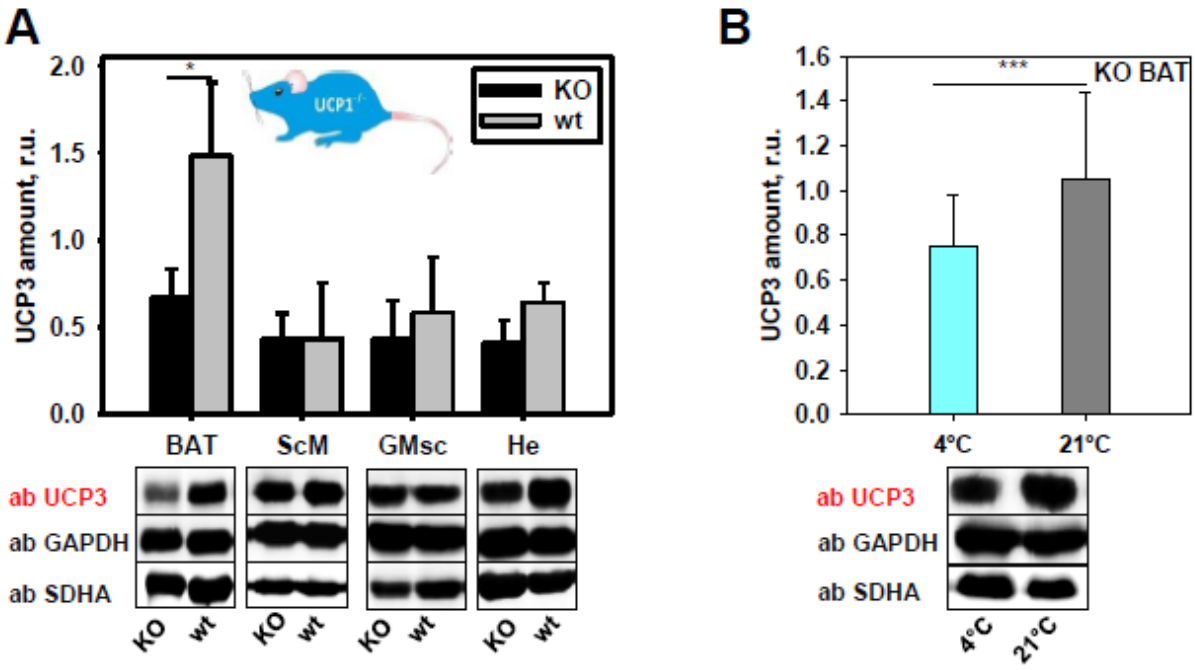

Figure 4

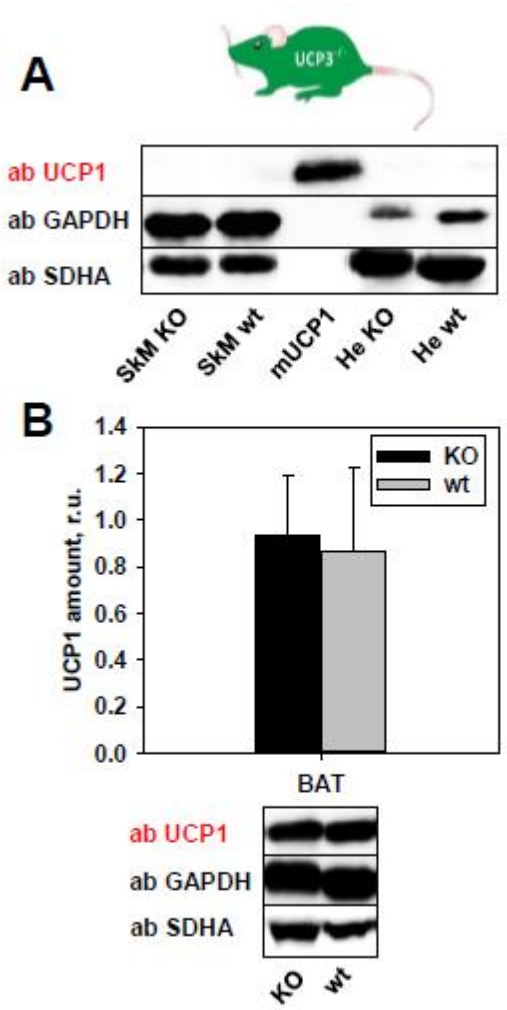

Figure 5

Conflict of Interest

The authors declare no conflict of interest.

Highlights

- The UCP3 amount in brown adipose tissue (BAT) is 0.51 ng/(μg total protein) and is nearly one order of magnitude higher than it is in muscles and the heart.
- The abundances of UCP3 and UCP1 in BAT are directly correlated, although the UCP3 amount is nearly 400 times lower than the UCP1 amount.
- The results imply that the specific UCP3 transport function is coherent with the proton transport function of highly abundant UCP1.