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Selective detection of UCP 3 expression in skeletal muscle: effect of thyroid status and temperature acclimation

O. Cunningham[a], A.M. McElligott[a], A.M. Carroll[a], E. Breen[a], C. Reguenga[b], M.E.M. Oliveira[b], J.E. Azevedo[b], R.K. Porter[a,*]

[a] *Department of Biochemistry, Trinity College Dublin, Biotechnology Building, Room 0.16, Dublin 2, Ireland*
[b] *Instituto de Biologia Molecular e Celular and Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Oporto, Portugal*

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Abstract

A novel peptide antibody to UCP 3 is characterized which is sensitive and discriminatory for UCP 3 over UCP 2, UCP 1 and other mitochondrial transporters. The peptide antibody detects UCP 3 expression in *E. coli*, COS cells and yeast expression systems. The peptide antibody detects a single ~ 33 kDa protein band in mitochondria from isolated rat skeletal muscle, mouse and rat brown adipose tissue, and in whole muscle groups (soleus and extensor digitorum longus) from mice. No 33 kDa band is detectable in isolated mitochondria from liver, heart, brain, kidney and lungs of rats, or gastrocnemius mitochondria from UCP 3 knock-out mice. From our data, we conclude that the peptide antibody is detecting UCP 3 in skeletal muscle, skeletal muscle mitochondria and brown adipose tissue mitochondria. It is also noteworthy that the peptide antibody can detect human, mouse and rat forms of UCP 3. Using the UCP 3 peptide antibody, we confirm and quantify the increased (2.8-fold) UCP 3 expression observed in skeletal muscle mitochondria isolated from 48-h-starved rats. We show that UCP 3 expression is increased (1.6-fold) in skeletal muscle of rats acclimated over 8 weeks to 8 °C and that UCP 3 expression is decreased (1.4-fold) in rats acclimated to 30 °C. Furthermore, UCP 3 expression is increased (2.3-fold) in skeletal muscle from hyperthyroid rats compared to euthyroid controls. In addition, we show that UCP 3 expression is only coincident with the mitochondrial fraction of skeletal muscle homogenates and not peroxisomal, nuclear or cytosolic and microsomal fractions.

Keywords: Uncoupling protein; UCP 3; Peroxisome; Mitochondria; Antibody; Thyroid; Skeletal muscle

1. Introduction

The function of the novel uncoupling proteins UCP 2 and UCP 3 is still a matter of contention. It has been reported that UCP 2 and UCP 3 act as uncoupling proteins, in the same manner as UCP 1, being conduits for proton translocation across the mitochondrial inner membrane [1]. Other data suggest that UCP 2 and UCP 3, like UCP 1, flip fatty acid anions across the mitochondrial inner membrane thus completing a cycle of protonated fatty acid diffusion and unprotonated fatty acid flipping leading to uncoupling [2]. In contrast, other data from isolated mitochondrial studies show no correlation between increased expression of UCP 2 and UCP 3 and increases in mitochondrial proton leak [3]. Dulloo et al. [4] have demonstrated a correlation between levels of UCP 3 transcript and fatty acid oxidation. Other evidence suggests that UCP 1, UCP 2 and UCP 3 facilitate superoxide translocation across the mitochondrial inner membrane [5]. UCP 2 knock-out mice have no obvious whole body metabolic phenotype to distinguish them from wild-type mice. However, UCP 2 knock-out mice do have an increased resistance to infection by *Toxoplasma gondii*. This resistance to infection has been attributed to increased reactive oxygen species (ROS) production by mitochondria in their macrophages [6]. Similarly, UCP 3 knock-out mice have no obvious phenotype to distinguish them from wild-

Abbreviations: ATCC, American Tissue Culture Center; BSA, bovine serum albumin; COIII, cytochrome oxidase subunit 3; cyt c, cytochrome *c*; ECL, enhanced chemiluminescence; EDL, extensor digitorum longus; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactopyranoside; OD, optical density; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SDS, sodium dodecylsulfate; STE, sucrose Tris EGTA buffer; UCP, uncoupling protein

* Corresponding author. Tel.: +353-1-6081617; fax: +353-1-6772400.
E-mail address: rkporter@tcd.ie (R.K. Porter).

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type mice [7,8]. However, decreased proton leak has been observed in isolated skeletal muscle mitochondria from UCP 3 knock-out mice [8]. By contrast, overexpression of UCP 3 protein in skeletal muscle of mice causes hyperphagia and increases whole body metabolic rate and proton leak of mitochondria in skeletal muscle [9]. However, it is not clear whether the increased leak is attributable to an increased amount of protein in the inner membrane or whether it is a specific function of the UCP 3 protein.

Functional analysis aside, it is possible to get clues to the function of UCPs, and the context in which that function operates, by observing their pattern of expression under various physiological and pathological conditions. Detection of UCP transcript is useful but is not necessarily representative of expression of the functional entity, i.e. the protein. This has clearly been the case for UCP 2 where transcript studies suggested ubiquitous expression in many mammalian tissues [10], whereas protein detection studies suggest UCP 2 is confined to specific tissues, namely lung, spleen, kidney, stomach and white adipose tissue [11]. Few studies have focused on UCP 3 protein expression despite the fact that commercial peptide antibodies to UCP 3 are available from Calbiochem, Chemicon International and Lilly. Peptide antibodies to UCP 3 have also been developed in-house by a number of groups [7,12]. However, in most cases, the sensitivity and discriminatory nature of those antibodies to other UCPs, and mitochondrial transporters in general, have not been satisfactorily demonstrated [12].

Northern blot analysis and Western blot analysis using peptide antibodies to UCP 3, have shown that UCP 3 is found predominantly in skeletal muscle of mammals [10,13,14]. Furthermore, starvation has been shown to increase UCP 3 mRNA levels in mouse skeletal muscle [13] and UCP 3 protein expression in mitochondria from skeletal muscle of rats [3]. Short-term cold exposure also increases UCP 3 protein expression in skeletal muscle mitochondria [15]. UCP 3 mRNA has also been detected in brown adipose tissue [10].

In this study, a novel peptide antibody to UCP 3 is described that is sensitive and discriminatory for UCP 3 over UCP 2, UCP 1 and other mitochondrial transporters. The antibody detects UCP 3 in skeletal muscle and brown adipose tissue as predicted from UCP 3 transcript studies. Using the UCP 3 peptide antibody, we confirm and quantify the increased UCP 3 expression in skeletal muscle mitochondria isolated from starved rats. We show that this specific antibody is sensitive enough for use on whole muscle homogenates. We show that UCP 3 expression is increased in skeletal muscle of rats after long-term cold exposure and that UCP 3 expression is decreased in rats acclimated to 30 °C. We demonstrate that UCP 3 expression is increased in skeletal muscle mitochondria of hyperthyroid rats. Our data also show that UCP 3 expression is coincident with the mitochondrial fraction of skeletal muscle homogenates.

2. Materials and methods

2.1. Isolation of mitochondria from liver, kidney, lungs, brain, brown adipose tissue

Mitochondria were prepared essentially by the method of Chappell and Hansford [16]. Tissues were removed, weighed and placed in ice-cold sucrose Tris EGTA buffer (STE) buffer (250 mM Sucrose, 5 mM Tris–HCl, 2 mM ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), pH 7.4). Tissues were chopped finely using scissors and washed several times with STE buffer. The tissue was homogenised by hand using a Dounce homogeniser as follows: four passes with a pestle of 0.26 inch clearance followed by six passes with a pestle of 0.12 inch clearance. The homogenate was centrifuged at 800 × *g* for 3 min at 4 °C, pelleting blood and debris. The pellet was discarded and the supernatant was centrifuged at 12,000 × *g* for 10 min at 4 °C yielding a ‘‘mitochondrial’’ pellet. The mitochondrial pellet was resuspended in STE buffer and re-centrifuged as described above. The resulting mitochondrial pellet was resuspended in STE buffer to the desired concentration.

2.2. Isolation of heart and skeletal muscle mitochondria

Skeletal muscle mitochondria from rat hind leg muscle and rat heart mitochondria were isolated by the method of Bhattacharya et al. [17]. Hindquarter muscle (all leg and a small amount of back muscle) and hearts were removed, weighed and placed in ice-cold isolation medium (100 mM sucrose, 9 mM ethylenediaminetetraacetic acid (EDTA), 100 mM Tris–HCl, 46 mM KCl, pH 7.4). Muscle and heart were finely chopped on a pre-cooled glass tile with a razor blade, added to isolation medium (200 ml) containing 0.5% (w/v) bovine serum albumin (BSA) and 0.02% (w/v) nagarse (Sigma, Protease VII) and incubated on ice, with stirring, for 5 min. The tissue was further disrupted using a Waring commercial blender at half-maximum speed for 15 min. The homogenate was centrifuged at 1500 × *g* for 3 min at 4 °C. The whitish lipid layer on top of the supernatant was removed using a spatula and the supernatant was centrifuged at 12,000 × *g* for 10 min at 4 °C. The mitochondrial pellet was resuspended in ice-cold isolation medium and centrifuged as above. The final mitochondrial pellet was resuspended in ice-cold isolation medium to the desired concentration.

2.3. Preparation of muscle groups for analysis

The hind limb muscles soleus and extensor digitorum longus (EDL) were removed, ensuring complete removal of associated tendons and connective tissue and placed in ice-cold muscle isolation medium (100 mM sucrose, 9 mM EDTA, 1 mM EGTA, 46 mM KCl, 100 mM Tris–HCl, pH 7.5). Muscles were finely chopped on a pre-cooled glass tile

and frozen in approximately 10 ml of liquid nitrogen in a porcelain mortar. A porcelain pestle was used to grind the frozen muscle to a fine powder. Once ground, EDL and muscles from each animal were individually resuspended in 500 μl of cold muscle isolation medium. Samples were aliquoted in 100 μl volumes and stored at −20 °C.

2.4. Subcellular fractionation of muscle and liver tissue

Fractionation of skeletal muscle was performed essentially as described for fractionation of liver by Hartl et al. [18] with minor modifications from Gouveia et al. [19] using minced muscle or liver from 48-h-starved Wistar rats.

2.5. Expression of protein and isolation of inclusion bodies from E. coli

A colony of *E. coli* cells, BL21 pLysS, which had been transfected with the appropriate plasmid (pET 3a-haUCP 1, pET 21d-hUCP 2 or pET 21d-hUCP 3) containing cDNA for the UCP required was inoculated into 5 ml of LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, 100 mg ampicillin in 1 l). The colony was amplified overnight and the following day, the culture was diluted to 500 ml and grown until OD_{600} had reached 0.4. Induction of expression of the UCP was carried out with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Two hours following the addition of IPTG, the cells were harvested at $10{,}000 \times g$ and the bacterial pellets frozen at −20 °C for at least an hour. Pellets were then thawed and inclusion bodies were isolated as described in Echtay et al. [20].

2.6. Expression of UCP 3 in yeast

The human UCP 3 yeast expression vector pYU3HSL was a gift from Dr. Daniel Ricquier (Centre de Recherche sur l'Endocrinologie Moleculaire et le Developpement, Meudon, France). The UCP 3 transcript was excised from pYU3HSL and subcloned into the yeast expression vector pYES2 (Invitrogen). The *Saccharomyces cerevisiae* strain INVSC1 (Invitrogen) was transformed with either the UCP 3 containing pYES2 expression vector or pYU3HSL using the *S.c.* EasyComp™ Kit (Invitrogen). Empty pYES was also transformed as a negative control. UCP 3 was expressed in yeast as described by Zhang et al. [21] with modifications. In brief, transformants were selected on SC-ura plates and single colonies were grown to an OD_{600}>2 in a preculture of SC-ura medium (0.67% yeast nitrogen base, 0.1% KH_2PO_4, 0.12% $(NH_4)_2SO_4$, 0.1% glucose, 2% lactate, 0.1% casamino acids, 20 mg/l tryptophan, 40 mg/l adenine). The yeast were diluted in 100 ml of YPL medium (1% yeast extract, 2% bactopeptone, 3% lactate, pH 4.5 with 10 M NaOH), to OD_{600} of 0.04. The yeast were grown at 30 °C with vigourous shaking to an OD_{600} ~ 1.5, at which time 1% galactose was added and the cells were harvested after a further 8 h. Following harvesting, the yeast were incubated with zymolyase (Sigma) until >90% spheroplast formation. The spheroplasts were resuspended in homogenisation buffer (0.6 M mannitol, 10 mM Tris–malate, pH 7.4, 0.1% BSA, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF) and homogenised using a Dounce homogeniser.

2.7. COS cell expression of UCP 3

The UCP 3 transcript was excised from pYU3HSL and subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen). COS-1 cells were obtained from the American Tissue Culture Center (ATCC). All cell culture reagents were supplied by GIBCO (Invitrogen). COS-1 cells were seeded in six-well plates at ~ 2×10^5 cells per well and grown overnight at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 U/ml penicillin, 100 mg/ml streptomycin sulfate and 10% foetal calf serum under 95% air/5% CO_2, then transiently transfected with pcDNA3.1 containing the UCP 3 transcript and pcDNA3.1 alone using Fugene (Roche) according to the manufacturers instructions. Twenty-four hours following transfection 100 μl of sodium dodecylsulfate (SDS) sample buffer (0.0625 M Tris–HCl, pH 6.8, 10% (w/v) glycerol, 4% (w/v) SDS, 5% (w/v) β-mercaptoethanol and 0.005% (w/v) bromophenol blue) was added to the wells and the cells were homogenised by being drawn up repeatedly through a narrow bore needle. The cell homogenates were stored at −20 °C until analysis.

COS-1 cells were also transfected with pcDNA3.1 containing the UCP 3 transcript and stable clones were isolated using neomycin selection. Colonies were isolated when they had grown to ~ 2 mm in diameter and expanded in selection media. Stable clones were harvested in SDS sample buffer as described above. Stable clones with the pcDNA3.1 vector alone were also isolated for use as a negative control.

2.8. Protein determination

Tissue and mitochondrial protein concentration was determined according to the procedure described by Markwell et al. [22].

2.9. Preparation of samples for polyacrylamide gel electrophoresis

Samples (10–100 μg protein) were solubilised in sample buffer; 0.0625 M Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 1.66% β-mercaptoethanol (added directly prior to use) and 0.001% bromophenol blue. Prior to loading on gel, samples were vortexed and pulsed in a minifuge before boiling at 100 °C for 5 min.

2.10. Polyacrylamide gel electrophoresis

Gels were run using the Laemmli [23] system for denaturing gels. Slight modifications were used as de-

scribed: 0.375 M Tris–HCl, pH 8.7 and 0.1% SDS were used in all solutions, except for the gels for the subcellular fractionation and peroxisome samples, which had 0.75 M Tris. Linear 12% polyacrylamide gels were prepared from a stock solution containing 30% (w/v) acrylamide; 0.4% (w/v) bis-acrylamide. Samples were prepared using Laemmli sample buffer containing either 5% (w/v) β-mercaptoethanol or 20–100 mM dithiothreitol, and subsequently incubated for 5 min at 100 °C or 20 min at 70 °C. The gel dimensions, for the subcellular fractionation and peroxisome samples, were 14 cm × 10 cm × 1 mm. Gels for whole muscle samples were 9 cm × 7 cm × 1.5 mm. All other gels were 9 cm × 7 cm × 0.75 mm. All gels were run on a Protean II or Protean III (Bio-Rad) gel system.

2.11. Western blotting analysis

Following polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose or polyvinylidene difluoride (PVDF) (Millipore). Transfer was achieved using a semi-dry apparatus for 2 h at 110 mA or a Bio-Rad Trans-Blot Transfer Cell for 1–4 h at 20 V/cm. After transfer was complete, blots were blocked for 1 h in phosphate-buffered saline (PBS)-Tween (0.14 M NaCl, 2.7 mM KCl, 11.5 mM Na_2PO_4, 1.8 mM KH_2PO_4, pH 7.4 with 0.1% Tween-20) containing 5% non-fat dried milk (Marvel). This was followed by three 10-min washes with PBS-Tween.

Blots were probed with primary antibody overnight at 4 °C in the same solution containing 1:5000 dilution of an affinity-purified anti-UCP 3 peptide antibody, a 1:5000 dilution of an affinity-purified anti-UCP 2 peptide antibody, a 1:5000 dilution of an affinity-purified anti-UCP 1 peptide antibody, a 1:1000 dilution of rabbit antiserum directed to human Pex14p [24], a 1:5000 dilution of a monoclonal antibody directed to the α-subunit of the mitochondrial ATP synthase (clone 7H10-BD4, Molecular Probes), a 1:500 dilution of purified mouse anti-cytochrome *c* monoclonal antibody (BD PharMingen), a 1:1000 dilution of peptide antibody to subunit III of cytochrome oxidase or a 1:10,000 dilution of the full-length UCP 1 antibody. These steps were followed by three 10-min washes in PBS-Tween.

Blots were then probed with secondary antibody incubated in 1:10,000 dilution of a goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (for cytochrome *c*), a donkey anti-sheep IgG HRP conjugate (for full-length UCP 1 antibody) or a goat/donkey anti-rabbit IgG HRP conjugate (for all others) secondary antibody in PBS-Tween containing 5% non-fat dried milk for 1 h. After a further three 10-min washes in PBS-Tween, the blots were developed using an enhanced chemiluminescence (ECL) detection system (Amersham-Biosciences) and visualized by exposure to X-ray film. The ATP synthase ($F_1\alpha$) and Pex14p blots were developed using chloronaphthol (Sigma) and hydrogen peroxide as substrates for the HRP-linked secondary antibody. The antibody to the $F_1\beta$ was raised by the β-subunit of the ATP-synthase from *Neurospora crassa*.

2.12. Densitometry

Following Western blot development, the relative abundance of UCP 3 was determined by using densitometry. The band intensities of the exposed film were analysed using Scion Imaging Software (Scion Corporation, Maryland, USA).

2.13. UCP antibodies

Peptide synthesis, conjugation of peptides to BSA and injection of rabbits was undertaken by Eurogentec (Parc Scientifique du Sart Tilman, 4102 Seraing, Belgium). Polyclonal antibodies in the resulting rabbit anti-sera were affinity-purified on a protein A column. The peptides to which polyclonal antibodies were raised are as follows: UCP 1, 145...CHLHGIKPRYTGYNA...158; UCP 2, 147...QARAGGGRRYQSTVEA...162; UCP 3, 141...TGGERKYRGTMDAYRC...156. The full-length antibody to UCP 1 was a gift from Daniel Ricquier (Centre de Recherche sur l'Endocrinologie Moleculaire et la Developpement).

3. Results

In a bid to distinguish between the uncoupling proteins UCP 1, UCP 2 and UCP 3, antibodies were raised to peptide sequences unique to each uncoupling protein based on similarities with the rat sequences. The resulting affinity-purified polyclonal antibodies were tested for their ability to detect UCP 1, 2 and 3 protein expressed by *E. coli* (Fig. 1). Blot A shows that anti-UCP 3 peptide antibodies detected only UCP 3 (lane 1) and not UCP 2 (lane 2) or UCP 1 (lane 3). Blot B demonstrates that UCP 2 antibodies could not discriminate between the UCPs and detected UCP 3 (lane 1), UCP 2 (lane 2) and UCP 1 (lane 3). Blot C shows that the UCP 1 antibodies only detected UCP 1 (lane 3) and not UCP 2 (lane 2) or UCP 3 (lane 1). One general observation is that all expressed uncoupling proteins had apparent

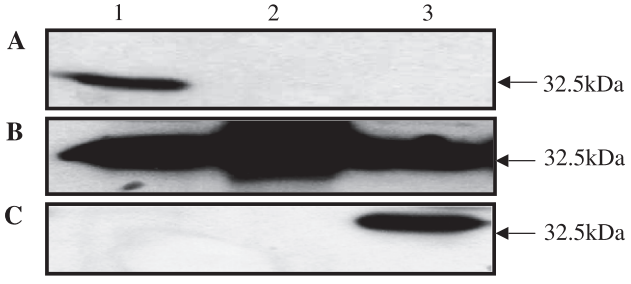

Fig. 1. Anti-UCP 3 peptide antibody is specific for *E. coli* expressed UCP 3. Lane 1, 5 μg of hUCP 3; lane 2, 20 μg hUCP 2; lane 3, 20 μg haUCP 1. Polyclonal peptide antibodies to rUCP 3 (Blot A), rUCP 2 (Blot B) and rUCP 1 (Blot C) were used at a dilution of 1:5000. Exposure time was the same for all samples (30 s).

molecular masses circa 32–34 kDa. All blots were exposed for equal time periods (30 s).

For the purpose of estimating increases in UCP 3 protein expression, we were able to show that there is a linear relationship between the amount of *E. coli* expressed UCP 3 and the resultant intensity of the bands on the exposed film of a given immunoblot (Fig. 2).

In addition to detection of UCP 3 expressed in *E. coli*, the polyclonal peptide antibodies to UCP 3 were able to detect UCP 3 transiently expressed in mammalian COS cells (Fig. 3A) using 10^6 (lane 1) and 10^5 (lane 2) cells. No protein was detected in COS cells transfected with an empty vector (lane 3). As might be expected, the polyclonal peptide antibodies to UCP 1 could not detect anything in mammalian COS cells transiently expressing UCP 3 at either cell concentration (results not shown). Fig. 3B shows that the polyclonal peptide antibodies to UCP 3 were able to detect UCP 3 expressed in a stably transfected clone of mammalian COS cells without having to isolate mitochondria (lane 1). Anti-UCP 2 or anti-UCP 1 peptide antibodies did not detect this band (results not shown). No protein was detected in control cells (lane 2).

UCP 3 was also expressed in yeast cells (Fig. 3C). The polyclonal peptide antibodies to UCP 3, detected a ~ 33 kDa band in cell homogenate containing plasmid expressing UCP 3 (lane 1) but detected nothing in yeast control cell homogenate (lane 2).

Fig. 4A shows that our anti-UCP 3 antibody detected a 33 kDa band in yeast cells expressing UCP 3 (lane 1), in mitochondria isolated from skeletal muscle of non-starved rats (lane 4) (no particular muscle group selected), in mitochondria isolated from skeletal muscle of starved rats (lane 5) and in mitochondria from brown adipose tissue of cold-adapted rats (lane 7). No UCP 3 was detected in rat liver mitochondria (lane 2), rat heart mitochondria (lane 3), mitochondria from rat brain (lane 6), mitochondria from rat kidney (lane 8) or mitochondria from rat lung (lane 9). Although 100 μg of mitochondria were used in the blots in Fig. 4, the anti-UCP 3 peptide antibody will detect UCP 3 at total skeletal muscle mitochondrial protein concentrations of

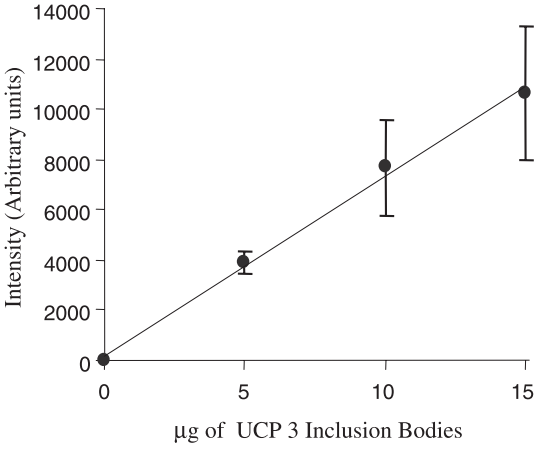

Fig. 2. UCP 3 immunoblot detection intensity as a function of amount of expressed UCP 3. Antibody to UCP 3, at a dilution of 1:5000, was used for *E. coli*-expressed inclusion bodies over a range of concentrations detected (0, 5, 10, and 15 μg). The relative abundance of UCP 3 was determined using densitometry. The band intensities of the exposed film were analysed using Scion Imaging. Intensity is measured in arbitrary units. The graph represents data for duplicate samples at each concentration (mean ± range). The line was fitted using regression analysis ($R = 0.999$).

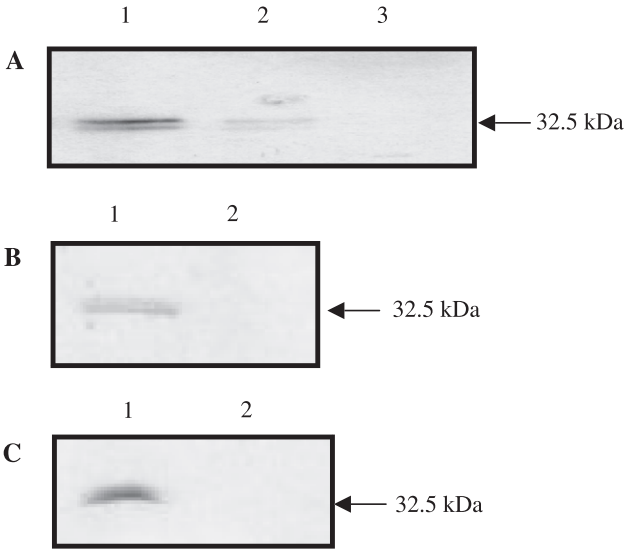

Fig. 3. Anti-UCP 3 peptide antibody detects UCP 3 expressed in mammalian and yeast cells. Blot A: lane 1, COS cells (~ 10^6 cells) transiently expressing UCP 3; lane 2, COS cells (~ 10^5 cells) transiently expressing UCP 3; lane 3, COS cells (~ 10^6 cells) containing empty vector. Blot B: lane 1, stable transfects of COS cells (~ 10^6 cells); lane 2, non-transfected COS cells (~ 10^6 cells). Blot C: lane 1, yeast cells (50 μg) transfected with pYES vector expressing UCP 3; lane 2, yeast cells (50 μg) transfected with pYES vector without UCP 3. Anti-UCP 3 peptide antibodies were used at a dilution of 1:5000. Only a single band was detected using the anti-UCP 3 antibody.

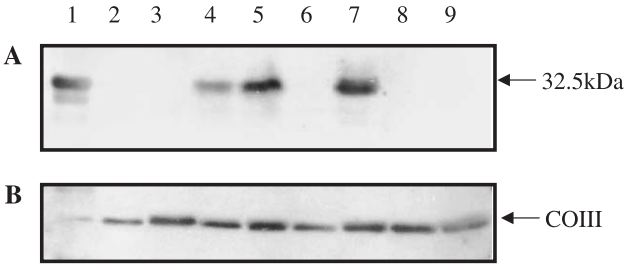

Fig. 4. Anti-UCP 3 peptide antibody detects UCP 3 in rat skeletal muscle mitochondria and brown adipose tissue. (A) UCP 3 peptide antibody and (B) antibody to cytochrome oxidase subunit III to: lane 1, yeast (~ 50 μg) expressing UCP 3; lane 2, liver mitochondria (100 μg) from non-starved rats; lane 3, heart mitochondria from non-starved rats (100 μg); lane 4, skeletal muscle mitochondria (100 μg) from non-starved rats; lane 5, skeletal muscle mitochondria (100 μg) from 48-h-starved rats; lane 6, rat mitochondria from whole brain (100 μg); lane 7, brown adipose tissue mitochondria from rats acclimated to the cold (8 ± 2 °C) for 8 weeks (100 μg); lane 8, rat kidney mitochondria (100 μg); lane 9, rat lung mitochondria (100 μg).

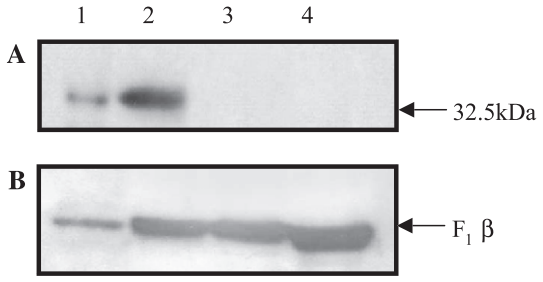

Fig. 5. Anti-UCP 3 peptide antibody detects UCP 3 in gastrocnemius mitochondria from wild-type but not UCP 3 knock-out mice. Immunoblots of UCP 3 peptide antibody (Blot A) and antibody to the β-subunit of the F_1-ATP synthase (Blot B): lane 1, yeast (~ 50 μg) expressing UCP 3; lane 2, gastrocnemius muscle mitochondria from C57BL/6J wild-type (WT) 6-week-old female mice; lane 3, gastrocnemius muscle mitochondria from C57BL/6J UCP 3 knock-out 6-week-old female mice; lane 4, rat liver mitochondria (100 μg).

50 μg from non-starved rats (results not shown). Fig. 4B gives a relative measure of the amount of mitochondrial protein loaded in each lane.

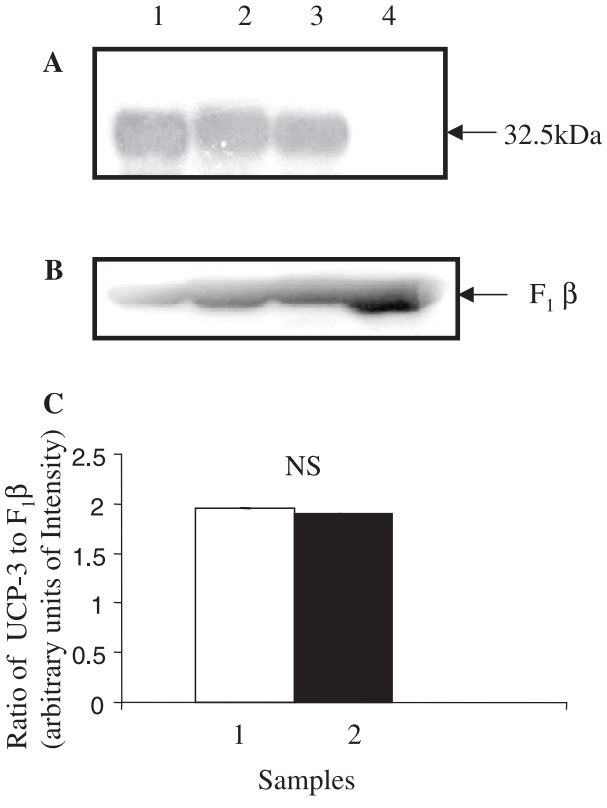

Fig. 6. Anti-UCP 3 peptide antibody detects equivalent amounts of UCP 3 in brown adipose tissue mitochondria from wild-type and UCP 1 knock-out mice. Immunoblots of UCP 3 peptide antibody (Blot A) and antibody to the β-subunit of the F_1-ATP synthase (Blot B): lane 1, yeast (~ 50 μg) expressing UCP 3; lane 2, brown adipose tissue mitochondria from C57BL/6J wild-type (WT) mice (100 μg); lane 3, brown adipose tissue mitochondria from C57BL/6J UCP 1 knock-out mice (100 μg); lane 4, mouse liver mitochondria (100 μg). The relative abundance of UCP 3 in brown adipose tissue mitochondria from UCP 1 wild-type (sample 1) and UCP 1 knock-out mice (sample 2) is given in panel C (mean ± S.E.M.). (NS, not significant).

The primary in vivo site of UCP 3 mRNA and protein expression is skeletal muscle. In Fig. 5, the peptide antibody to UCP 3 detects a 33 kDa protein in the yeast expressing UCP 3 (lane 1) and in mitochondria isolated from the gastrocnemius of wild-type mice (lane 2). However, no 33 kDa protein is detected in mitochondria isolated from the gastrocnemius of UCP 3 knock-out mice (lane 3) or in rat liver mitochondria (lane 4).

The most abundant UCP in brown adipose tissue would be UCP 1 [10]. Therefore, we wished to reassure ourselves that our UCP 3 antibody was not detecting native UCP 1. Fig. 6A and B demonstrate that the intensity of UCP 3 detection is not different when comparing brown adipose mitochondria from wild-type (lane 2) and UCP 1 knock-out mice (lane 3) and this is demonstrated graphically (Fig. 6C). As expected, UCP 3 was detected in yeast expressing UCP 3 (lane 1) but not in mouse liver mitochondria (lane 4).

The immunoblot in Fig. 7A shows that expression of UCP 3 protein in rat skeletal muscle is sensitive to temperature acclimation of the animal. As previously demonstrated, UCP 3 is detectable using the polyclonal anti-UCP 3 peptide antibody in yeast cells expressing UCP 3 (lane 1) and in mitochondria from non-starved rats housed at a constant 20 °C room temperature (lane 2). Skeletal muscle

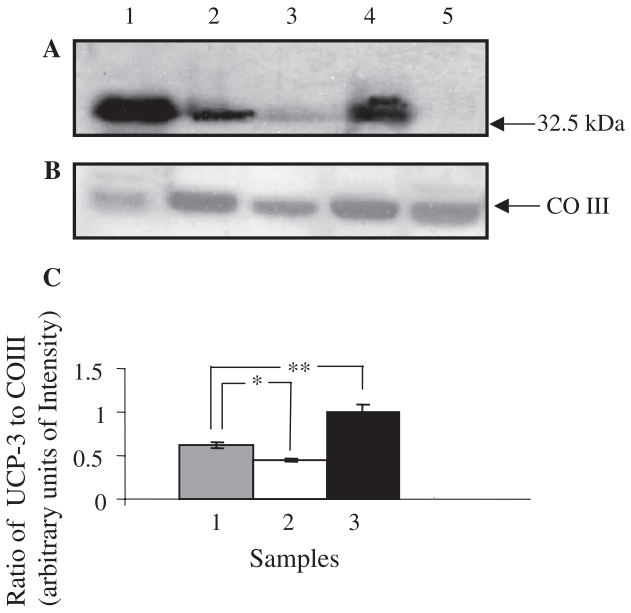

Fig. 7. The effect of temperature acclimation of rats on UCP 3 expression in skeletal muscle mitochondria. (A) UCP 3 peptide antibody and (B) antibody to cytochrome oxidase subunit III to: lane 1, yeast expressing UCP 3 (~ 50 μg); lane 2, skeletal muscle mitochondria from rats kept at 20 °C (100 μg); lane 3, skeletal muscle mitochondria (100 μg) from rats exposed to 30 ± 2 °C for 8 weeks; lane 4, skeletal muscle mitochondria from rats exposed to the cold (8 ± 2 °C) for 8 weeks (100 μg); lane 5, liver mitochondria (100 μg) from rats at 20 ± 2 °C for 8 weeks. (C) The ratio of UCP 3 protein expression to that of cytochrome oxidase, subunit 3 (COIII), for (sample 1) skeletal muscle mitochondria from rats acclimated to 20 °C, (sample 2) skeletal muscle mitochondria from rats acclimated to 30 °C and (sample 3) skeletal muscle mitochondria from rats acclimated to 8 °C. Data are from three separate preparations. *$P < 0.0047$ **$P < 0.0113$.

mitochondria isolated from rats acclimated to a temperature of 30 °C demonstrates a 3-fold decreased UCP 3 expression (lane 3) compared to those at 20 °C, while skeletal muscle mitochondria isolated from rats acclimated to a temperature of 8 °C for 8 weeks (lane 4) demonstrate increased UCP 3 expression compared to those at 20 °C (lane 2). As expected, no UCP 3 was detected in liver mitochondria (lane 5). The temperature acclimation experiments were repeated several times with similar results. Fig. 7B gives a relative measure of the amount of mitochondrial protein loaded in each lane and demonstrates that the amount of mitochondrial protein loaded in each lane cannot account for the differences observed in the amount of UCP 3 protein in lanes 1–4. In addition, there is a significant ($P<0.0047$; $n=3$) 1.4-fold decrease in UCP 3 expression observed in mitochondria isolated from skeletal muscle of rats acclimated to 30 °C when compared to mitochondria from rats acclimated to 20 °C and there is a significant ($P<0.0113$; $n=3$) 1.6-fold increase in UCP 3 expression observed in mitochondria isolated from skeletal muscle of rats acclimated to 8 °C when compared to mitochondria from rats acclimated to 20 °C (Fig. 7C).

Data in the literature show that thyroid hormone administration increases transcripts of UCP 3 in skeletal muscle of mice and rats [10]. Fig. 8A shows a representative set of samples where UCP 3 protein expression is increased in skeletal muscle mitochondria from hyperthyroid rats (lane 5) compared to euthyroid controls (lane 3), an increase comparable with that found in mitochondria from starved animals (lane 4). The peptide antibody to rat UCP 3 does not detect a 33 kDa band in liver mitochondria (lane 2) but detects UCP 3 expressed in yeast (lane 1) as expected. Fig. 8B shows a representative set of samples indicating that differences in UCP 3 expression (Fig. 8A) cannot be accounted for by differences in lane loading as indicated by cytochrome oxidase subunit 3 (COIII) detection (Fig. 8B). Fig. 8C gives the ratio of UCP 3 expression to COIII expression for four separate preparations determined in triplicate. The results show a 2.3-fold ($P<0.002$, $n=4$) increase in UCP 3 expression in the hyperthyroid state with respect to mitochondria from euthyroid animals. In addition, Fig. 8C shows a 2.8-fold ($P<0.001$, $n=4$) increase in UCP 3 expression in mitochondria from 48-h-starved animals with respect to mitochondria from euthyroid control rats.

The amino acid motif used to define mitochondrial (anion) transporters has also been found to occur in transporters of the peroxisomal membrane [25]. Thus it seemed possible that UCP 3 might also exist in peroxisomes. A subcellular fractionation of skeletal muscle from 48-h-starved rats was performed in order to separate peroxisomes (identified using anti-Pex14p) from mitochondria (identified using antibody to the anti-$F_1\alpha$ subunit of the ATP synthase) (Fig. 9). Pure rat liver peroxisomes (lanes 1 and 14) were also identified using the anti-Pex14p antibody (blot B) and despite a small amount of mitochondrial contamination (blot C), it can be seen that the UCP 3 antibody does not react

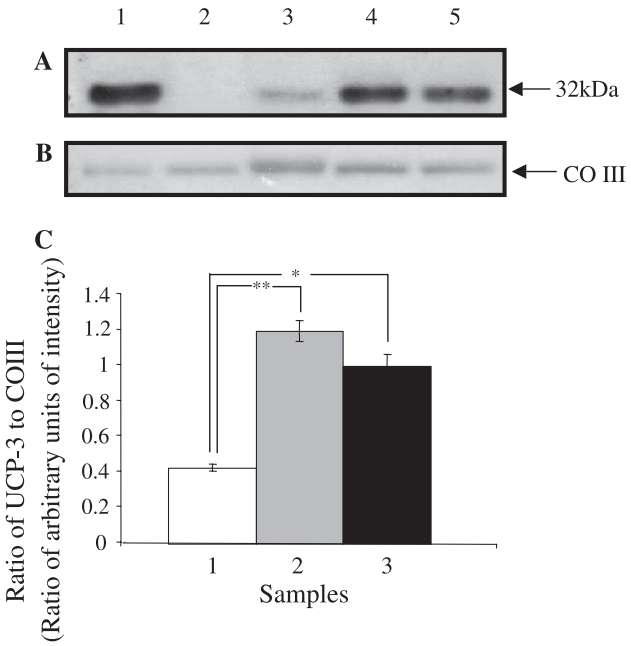

Fig. 8. The effect of thyroid status on UCP 3 expression levels in isolated mitochondria from rat skeletal muscle. Lane 1, 50 μg of mitochondria from yeast expressing UCP 3; lane 2, 100 mg of liver mitochondria from euthyroid rats; lane 3, 100 μg skeletal muscle from euthyroid rats; lane 4, 100 μg of skeletal muscle mitochondria from 48-h-starved rats; lane 5, 100 μg of skeletal muscle mitochondria from rats made hyperthyroid. Panel (A) is an immunoblot probed with a 1:5000 dilution of anti-UCP 3 peptide antibody. Panel (B) is an immunoblot probed with 1:1000 dilution of anti-COIII peptide antibody. Panel (C) shows the ratio of expression of UCP 3 to COIII for skeletal muscle mitochondria from euthyroid rats (sample 1), skeletal muscle mitochondria from 48-h-starved rats (sample 2) and skeletal muscle mitochondria from rats made hyperthyroid (sample 3). The graph represents data from four separate mitochondrial preparations each determined in triplicate. *$P<0.002$, **$P<0.001$.

with pure peroxisomes from rat liver (blot A). The UCP 3 antibody (blot A) detects protein in the supernatant from the $600\times g$ centrifugation of rat skeletal muscle homogenate (lanes 2 and 13), which is coincident with mitochondrial protein detection (blot C). However, peroxisomes are not detected in the $600\times g$ supernatant fraction from rat skeletal muscle homogenate (blot B). The pellet from the $600\times g$ centrifugation (cells and nuclei) (lanes 3 and 12) contains small amounts of UCP 3 (blot A), no peroxisomes (blot B) and some mitochondria (blot C). The resulting centrifugation of the $600\times g$ supernatant at $2350\times g$ resulted in a pellet termed heavy mitochondria (lanes 4 and 11) which contained a strong band for UCP 3 (blot A), a strong band for mitochondria (blot C) but no detection of peroxisomes (blot B). The supernatant from the centrifugation at $2350\times g$ was then centrifuged at $12{,}300\times g$ yielding a supernatant termed cytosolic and microsomal protein (lanes 5 and 10). A small amount of UCP 3 (blot A) and mitochondria (blot C) but no peroxisomes (blot B) were found in this fraction. The pellet from the $12{,}300\times g$ centrifugation contains a fraction termed light mitochondria (lanes 6 and 9). This fraction contains a lot of UCP 3 (blot

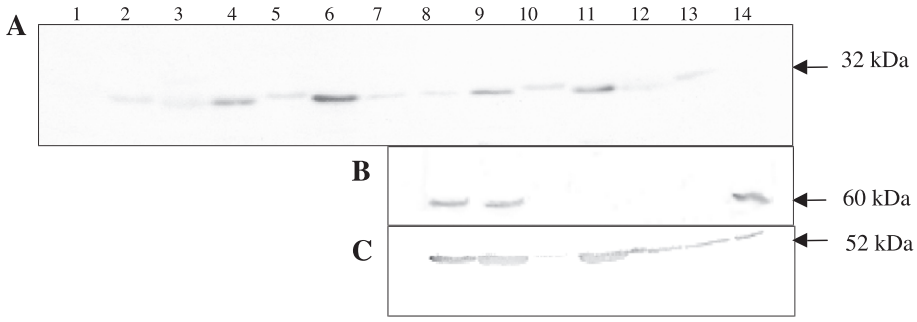

Fig. 9. Immunoblot of skeletal muscle tissue fractionation from 48-h-starved rats using anti-UCP 3 peptide antibody. Lanes 1 and 14, pure peroxisomes from rat liver. Lanes 5 and 10 cytosolic and microsomal proteins. Lanes 2 and 13, supernatant from a 600 × *g* centrifugation. Lanes 6 and 9, light mitochondria. Lanes 3 and 12, cells and nuclei. Lanes 7 and 8, pure peroxisomes. Lanes 4 and 11, heavy mitochondria. Blot A shows the distribution of UCP 3 using a 1:5000 dilution of anti-UCP 3 peptide antibody. Blot B shows the distribution of peroxisomes throughout the subcellular fractionation using a 1:1000 dilution of an antiserum directed to human Pex14p, a peroxisomal marker protein. Blot C shows the distribution of mitochondria throughout the subcellular fractionation using a 1:5000 dilution of a monoclonal antibody raised against the α-subunit of the mitochondrial F_1-ATPase.

A), a lot of peroxisomes (blot B) and a lot of mitochondria (blot C). Attempts to purify peroxisomes from this fraction using discontinuous Nycodenz density gradient centrifugation were not very successful. As shown in Fig. 9, no major enrichment of Pex14p can be observed in the organelles obtained after this procedure (compare lanes 8 and 9, panel B). However, a significant depletion (ca. 50%) of both $F_1\alpha$ and UCP 3 in these organelles can be easily observed (compare lanes 8 and 9 of panels A and C). Taken together, these data suggest (indicate) that UCP 3 (like $F_1\alpha$) is a mitochondrial protein (or is not a peroxisomal protein). An equivalent tissue fractionation was performed on rat liver from non-starved rats but no UCP 3 was detectable in any fractionation (results not shown).

Fig. 10A demonstrates that the anti-UCP 3 antibody can detect UCP 3 in whole muscle homogenates without the need to isolate mitochondria. Lane 1 demonstrates the anti-UCP 3 antibody detecting UCP 3 in yeast cells expressing UCP 3. UCP 3 could be detected in mouse EDL muscle (lanes 2 and 3) a predominantly glycolytic muscle and in mouse soleus muscle (lanes 4 and 5) a predominantly oxidative muscle. In Fig. 8B, antibodies to cytochrome *c* were used to give a relative measure of mitochondria density in the muscle groups (lanes 2–5). This particular antibody to cytochrome *c* works well on mammalian tissue but is known not to detect cytochrome *c* in yeast, as exemplified in lane 1.

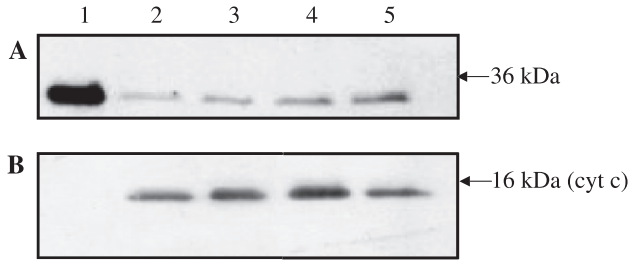

Fig. 10. UCP 3 detected in individual muscle groups (total muscle homogenates) from mouse. (A) 1:5000 dilution of UCP 3 antibody. (B) 1:5000 dilution of cytochrome *c* antibody. Lane 1, yeast expressing UCP 3. Lanes 2 and 3, extensor digitorum longus muscle (100 μg). Lanes 4 and 5, soleus muscle (75 μg). The cytochrome *c* antibody is known not to detect yeast cytochrome *c* (lane 1), but can detect mammalian cytochrome *c* (lanes 2–5).

Interestingly, the antibody to full-length UCP 1 detected a 33 kDa protein in cells transiently transfected with plasmid containing hUCP 3 and rat skeletal muscle mitochondria (results not shown).

4. Discussion

Although the function of UCP 3 is still contentious, clues to its physiological function can be obtained from observing their pattern of expression. As the protein is the functional entity, sensitive and discriminatory UCP detection antibodies are desirable. Northern blot analysis has shown that UCP 3 is expressed predominantly in skeletal muscle but also in brown adipose tissue [13]. However, limited immunodetection data exists for UCP 3. In this study, we demonstrate the selectivity and sensitivity of a peptide antibody to UCP 3.

Using uncoupling proteins purified from inclusion bodies expressed in *E. coli*, we showed that the anti-UCP 3 peptide antibody is selective for UCP 3 over UCP 2 and UCP 1 (Fig. 1) and that intensity of the UCP 3 bands correlates with abundance of UCP 3 protein (Fig. 2). In addition, the anti-UCP 1 antibody would also appear to be selective, detecting only *E. coli* expressed UCP 1 over UCP 2 and UCP 3 (Fig. 1).

The anti-UCP 3 peptide antibodies were also capable of detecting UCP 3 expressed in COS and yeast cells (Fig. 3). The same peptide antibody detected nothing in COS cells or yeast cells transfected with empty vector although both cell types contain mitochondria which themselves express transporters of an equivalent size. As might be

predicted, antibodies to the UCP 1 peptide antibody were unable to detect UCP 3 in these cells. But interestingly, antibodies to full-length UCP 1 were able to detect UCP 3 expressed in COS cells and in rat skeletal muscle mitochondria.

The pattern of the UCP 3 protein detection data (Fig. 4) are consistent with the UCP 3 mRNA detection data [10]. The antibody detected UCP 3 protein in skeletal muscle mitochondria and brown adipose tissue of rats. No UCP 3 was detected in mitochondria from tissues known to contain UCP 2 protein (kidney, brain and lungs) [11,26], nor was UCP 3 protein detected in heart or liver. The conclusion from these data is that the anti-UCP 3 antibody was detecting UCP 3 and not another member of the mitochondrial anion transporter family.

Our polyclonal UCP 3 peptide antibody can detect UCP 3 in mitochondria isolated from gastrocnemius of wild-type but not UCP 3 knock-out mice. Furthermore, in Fig. 6, it can be seen that the UCP 3 antibody detects UCP 3 to the same extent in brown adipose tissue of wild-type and UCP 1 knock-out mice. Taken together, it can be concluded that the anti-UCP 3 peptide antibody is selective for UCP 3 and is not detecting another member of the mitochondrial anion transport family.

Having established the discriminatory nature of the anti-UCP 3 peptide antibodies, we looked at the effect of long-term temperature acclimation on expression of native UCP 3. It was shown that cold-acclimated rats had increased (1.6-fold) UCP 3 expression compared to controls kept at room temperature (Fig. 7), whereas rats acclimated to 30 °C had decreased (1.4-fold) UCP 3. The suggestion is that UCP 3 protein expression is temperature-sensitive. These results again show our antibody to be sensitive to changes in the amount of UCP 3 expression per milligram of mitochondria. These results possibly contrast with those of Boss et al. [13] who showed that UCP 3 mRNA levels in mouse skeletal muscle do not increase with 48 h (short-term) cold exposure yet possibly compliment those of Simonyan et al. [15] who show that UCP 3 protein levels increase after 24 h (short-term) cold exposure.

Thyroid hormone is a major determinant of basal metabolism [27] and there are reports in the literature that thyroid hormone administration increases transcripts of UCP 3 in skeletal muscle of mice and rats [10]. In this study, we found that the hyperthyroid condition increases UCP 3 protein expression 2.3-fold (Fig. 8). The data are consistent with a fatty acid-dependent increase in proton leak in skeletal muscle mitochondria [28]. In addition, we were able to quantify (~ 2.8-fold) and confirm a previously observed [3] increase in UCP 3 protein expression in skeletal muscle mitochondria from starved rats. No increase in proton leak was observed in that instance [3]. In this context, as previously pointed out by others, one would expect to see an increase in UCP 3 levels upon starvation, as opposed to the observed decrease [3,13]. However, if UCP 3 has a role in fatty acid oxidation as suggested by Dulloo et al. [4], then increased UCP 3 expression on starvation would be consistent with that role.

Recent evidence demonstrated that transporters across the peroxisomal membrane also contain the 'mitochondrial' transporter motif [25]. However, subcellular fractionation of skeletal muscle from starved rats demonstrated that UCP 3 was exclusive to mitochondrial fractions (Fig. 9).

The sensitivity of the anti-UCP 3 antibody in detecting UCP 3 is further emphasized by the fact that there is no need to isolate mitochondria from muscle for UCP 3 to be detected. Our anti-UCP 3 antibody was able to detect UCP 3 associated with mitochondria in homogenates of mouse EDL muscle, a predominantly glycolytic muscle and mouse soleus muscle, a predominantly oxidative muscle. Although these particular results aren't novel, they confirm those of Giacobino [14] and compliment studies on glycolytic and oxidative muscle from humans [12].

In conclusion, our data shows that we have a peptide antibody raised to a rat UCP 3 sequence that is sensitive and discriminatory for UCP 3 over UCP 2, UCP 1 and other mitochondrial transporters. The UCP 3 peptide antibody also detects UCP 3 in isolated mitochondria from skeletal muscle and brown adipose tissue of rats and mice but not mitochondria isolated from UCP 3 knock-out mice. UCP 3 is also detectable using this antibody in whole mouse muscle homogenates without the need for isolation of mitochondria. We confirm and quantify the results of Cadenas et al. [3] that UCP 3 expression is increased in mitochondria isolated from starved animals. We show that UCP 3 expression is increased in cold-acclimated rats and decreased in rats acclimated to 30 °C, suggesting temperature-sensitive expression. In addition, we show that UCP 3 expression is thyroid-sensitive.

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References

[1] K.S. Echtay, E. Winkler, K. Frischmuth, M. Klingenberg, Uncoupling proteins 2 and 3 are highly active H(+) transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone), Proc. Natl. Acad. Sci. 98 (2001) 1416–1421.

[2] M. Jabůrek, M. Vařecha, R.E. Gimeno, M. Dembski, P. Ježek, M. Zhang, P. Burn, L.A. Tartaglia, K.D. Garlid, Transport function and regulation of mitochondrial uncoupling proteins 2 and 3, J. Biol. Chem. 274 (1999) 26003–26007.

[3] S. Cadenas, J.A. Buckingham, S. Samec, J. Seydoux, N. Din, A.G. Dulloo, M.D. Brand, UCP 2 and UCP 3 rise in starved rat skeletal muscle but mitochondrial proton conductance is unchanged, FEBS Lett. 462 (1999) 257–260.

[4] A.G. Dulloo, S. Samec, J. Seydoux, Uncoupling protein 3 and fatty acid metabolism, Biochem. Soc. Trans. 29 (2001) 785–790.

[5] K.S. Echtay, D. Roussel, J. St-Pierre, M.B. Jekabsons, S. Cadenas, J.A. Stuart, J.A. Harper, S.J. Roebuck, A. Morrison, S. Pickering, J.C. Clapham, M.D. Brand, Superoxide activates mitochondrial uncoupling proteins, Nature 415 (2002) 96–99.

[6] D. Arsenijevic, H. Onuma, C. Pecqueur, S. Raimbault, B.S. Manning, B. Miroux, E. Couplan, M.-C. Alves-Guerra, R. Surwit, F. Bouillaud, D. Richard, S. Collins, D. Ricquier, Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production, Nat. Genet. 26 (2000) 435–439.

[7] A.J. Vidal-Puig, D. Grujic, C.-Y. Zhang, T. Hagen, O. Boss, Y. Ido, A. Szczepanik, J. Wade, V. Mootha, R. Cortright, D.M. Muoio, B.B. Lowell, Energy metabolism in uncoupling protein 3 gene knockout mice, J. Biol. Chem. 275 (2000) 16258–16266.

[8] D.-W. Gong, S. Monemdjou, O. Gavrilova, L.R. Leon, B. Marcus-Samuels, C.J. Chou, C. Everett, L.P. Kozak, C. Li, C. Deng, M.E. Harper, M.L. Reitman, Lack of obesity and normal response to fasting and thyroid hormone in mice lacking uncoupling protein-3, J. Biol. Chem. 275 (2000) 16251–16257.

[9] J.C. Clapham, J.R. Arch, H. Chapman, A. Haynes, C. Lister, G.B. Moore, V. Piercy, S.A. Carter, I. Lehner, S.A. Smith, L.J. Beeley, R.J. Godden, N. Herrity, M. Skehel, K.K. Changani, P.D. Hockings, D.G. Reid, S.M. Squires, J. Hatcher, B. Trail, J. Latcham, S. Rastan, A.J. Harper, S. Cadenas, J.A. Buckingham, M.D. Brand, A. Abuin, Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean, Nature 406 (2000) 415–418.

[10] D. Ricquier, F. Bouillaud, The uncoupling protein homologues: UCP1, UCP2, UCP 3, StUCP and AtUCP, Biochem. J. 345 (2000) 161–179.

[11] C. Pecqueur, M.-C. Alves-Guerra, C. Gelly, C. Levi-Meyrueis, E. Couplan, S. Collins, D. Ricquier, F. Bouillaud, B. Miroux, Uncoupling protein 2, in vivo distribution, induction upon oxidative stress, and evidence for translational regulation, J. Biol. Chem. 276 (2001) 8705–8712.

[12] M.K.C. Hesselink, H.A. Keizer, L.B. Borghouts, G. Schaart, C.F.P. Kornips, L.J. Slieker, K.W. Sloop, W.H.M. Saris, P. Schrauwen, Protein expression of UCP 3 differs between human type 1, type 2a, and type 2b fibers, FASEB J. 15 (2001) 1071–1073.

[13] O. Boss, S. Samec, F. Kühne, P. Bijlenga, F. Assimacopoulos-Jeannet, J. Seydoux, J.-P. Giacobino, P. Muzzin, Uncoupling protein-3 expression in rodent skeletal muscle is modulated by food intake but not by changes in environmental temperature, J. Biol. Chem. 273 (1998) 5–8.

[14] J.-P. Giacobino, Uncoupling protein 3 biological activity, Biochem. Soc. Trans. 29 (2001) 774–777.

[15] R.A. Simonyan, M. Jimenez, R.B. Ceddia, J.-P. Giacobino, P. Muzzin, V.P. Skulachev, Cold-induced changes in the energy coupling and the UCP 3 level in rodent skeletal muscles, Biochim. Biophys. Acta 1505 (2001) 271–279.

[16] J.B. Chappell, R.G. Hansford, in: G.D. Birnie (Ed.), Subcellular Components: Preparation and Fractionation, Butterworths, London, 1972, pp. 77–91.

[17] S.K. Bhattacharya, J.H. Thakar, P.L. Johnson, D.R. Shanklin, Isolation of skeletal muscle mitochondria from hamsters using an ionic medium containing ethylenediaminetetraacetic acid and nagarse, Anal. Biochem. 192 (1991) 344–349.

[18] F.U. Hartl, W.W. Just, A. Koster, H. Schimassek, Improved isolation and purification of rat liver peroxisomes by combined rate zonal and equilibrium density centrifugation, Arch. Biochem. Biophys. 237 (1985) 124–134.

[19] A.M.M. Gouveia, C. Reguenga, M.E.M. Oliveira, C. Eckerskorn, C. Sá-Miranda, J.E. Azevedo, Alkaline density gradient floatation of membranes: polypeptide composition of the mammalian peroxisomal membrane, Anal. Biochem. 724 (1999) 270–277.

[20] K.S. Echtay, Q. Liu, T. Caskey, E. Winkler, K. Frischmuth, M. Bienengraber, M. Klingenberg, Regulation of UCP 3 by nucleotides is different from regulation of UCP1, FEBS Lett. 450 (1999) 8–12.

[21] C.-Y. Zhang, T. Hagen, V.K. Mootha, L.J. Slieker, B.B. Lowell, Assessment of uncoupling activity of the human uncoupling protein 3 short form and three mutants of the uncoupling protein gene using a yeast heterologous expression system, FEBS Lett. 449 (1999) 129–134.

[22] M.A. Markwell, S.M. Haas, L.L. Bieber, N.E. Tolbert, A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples, Anal. Biochem. 87 (1978) 206–210.

[23] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[24] C. Reguenga, M.E. Oliveira, A.M. Gouveia, C. Sá-Miranda, J.E. Azevedo, Characterization of the mammalian peroxisomal import machinery: Pex2p, Pex5p, Pex12p, and Pex14p are subunits of the same protein assembly, J. Biol. Chem. 276 (2001) 29935–29942.

[25] F.E. Weber, G. Minestrini, J.H. Dyer, M. Werder, D. Boffelli, S. Compassi, E. Wehrli, R.M. Thomas, G. Schulthes, H. Hauser, Molecular cloning of a peroxisomal Ca^{2+}-dependent member of the mitochondrial carrier superfamily, Proc. Natl. Acad. Sci. 94 (1997) 8509–8514.

[26] K.S. Echtay, M.D. Brand, Coenzyme Q induces GDP-sensitive proton conductance in kidney mitochondria, Biochem. Soc. Trans. 29 (2001) 763–768.

[27] M.D. Brand, L.-F. Chien, E.K. Ainscow, D.F.S. Rolfe, R.K. Porter, The causes and functions of mitochondrial proton leak, Biochim. Biophys. Acta 1187 (1994) 132–139.

[28] A. Lanni, L. Beneduce, A. Lombardi, M. Moreno, O. Boss, P. Muzzin, J.P. Giacobino, F. Goglia, Expression of uncoupling protein-3 and mitochondrial activity in the transition from hypothyroid to hyperthyroid state in rat skeletal muscle, FEBS Lett. 444 (1999) 250–254.