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Rapid report

Images of mitochondrial UCP 1 in mouse thymocytes using confocal microscopy

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Abstract

The aim of this study was to demonstrate the constitutive expression of mitochondrial uncoupling protein 1 (UCP 1) in pure thymocytes using laser scanning confocal microscopic imagery. To that end we probed thymocytes from UCP 1 knock-out and wild-type mice. Mitochondrial location in thymocytes was determined using Mitotracker Red and the nucleus was labelled using Hoescht stain. We demonstrate that all cells investigated were thymocytes as determined by a monoclonal antibody specific for the thymocyte surface marker Thy 1 (CD90) pre-coupled to a fluorescent labelled (Alexa 448, green). Using a primary peptide antibody specific to UCP 1, and secondary fluorescently labelled (Alexa 647, magenta) antibody, we were able to demonstrate that UCP 1 is associated with mitochondria in thymocytes from UCP 1 wild-type mice but not thymocytes from UCP1-knock-out mice. These are the first images demonstrating the presence of UCP 1 in thymocyte mitochondria, in situ, and the first to clearly demonstrate UCP 1 expression in cells other than brown adipocytes. We conclude that mouse thymocytes contain UCP 1 in their mitochondria.

Keywords: Uncoupling protein-1; Thymus; Thymocytes; Mitochondria; UCP 1 knock-out mice; Confocal microscopy

1. Introduction

Until recently, mitochondrial uncoupling protein 1 (UCP 1) has only ever been associated with brown adipose tissue (BAT) [1–3].

However, extensive direct empirical evidence for the detection of UCP 1 in thymocytes was recently demonstrated in our laboratory [4–6]. The thymus is the site of T-helper and cytotoxic T-cell maturation [7]. The thymus is involved in determining self from non-self and its size and activity is greatest in younger mammals. Evidence from Carroll et al. [5] for the existence of UCP 1 in thymus includes (i) reverse transcriptase-polymerase chain reaction detection of RNA transcripts for UCP 1 in whole thymus and in isolated thymocytes of rats and mice (Note, isolated thymocytes were not contaminated with brown adipocytes, but contained >99% Thy-1 (CD90) positive lymphocytes as identified by FACS analysis), (ii) peptide antibodies specific for UCP 1, detected protein of appropriate molecular mass in mitochondria isolated from whole thymus and thymocytes of rats and mice, but not in thymus mitochondria from UCP 1 knock-out mice and (iii) immunodetection of UCP 1 in thymus mitochondria was achieved using another characterized [8] polyclonal anti-UCP 1 peptide antibody and a polyclonal antibody to the full-length UCP 1 protein [5].

Interestingly, Frontini et al. [9] have recently produced histological images of UCP 1 detection associated with BAT in the vicinity of thymus tissue from mouse/rat. These authors suggest that any detection of UCP 1 in thymus is solely due to associated BAT. It is also noteworthy that, reports of UCP 1 expression in uterine longitudinal smooth muscle cells by Nibbelink et al. [10] were later refuted by Rousset et al. [11] as being due to UCP 2. It should also be noted that although UCP 1 has been clearly demonstrated to be the key protein in BAT in the

Abbreviations: BAT, brown adipose tissue; BSA, bovine serum albumin; FBS, foetal bovine serum; PBS, phosphate buffered saline; UCP, uncoupling protein

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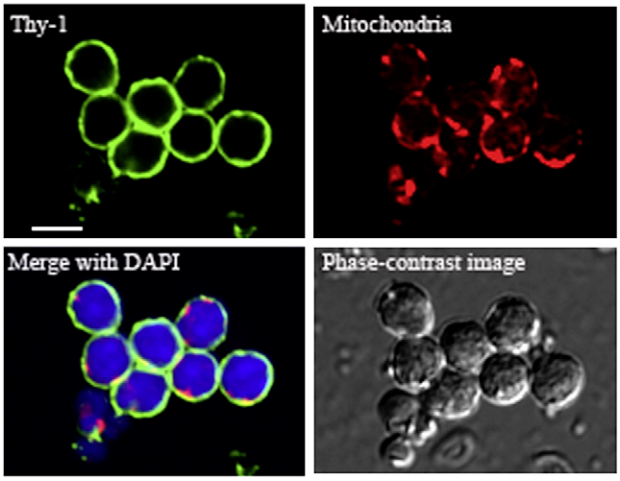

Fig. 1. Cellular markers of Thy-1 surface antigen, mitochondria and nucleus within thymocytes. Isolated thymocytes were incubated with Mitotracker Red for 60 min at 37 °C and fixed and permeabilized consecutively in methanol and acetone at −20 °C for 5 min each on poly-L-lysine slides and subsequently probed with the polyclonal anti-Thy-1 IgG. Thy-1 was detected using Alexa 488 (green). The nucleus was stained with Hoechst stain (blue). The merge image shows the large nucleus of thymocytes and the small cytoplasmic space between the nucleus and the plasma membrane, in which the mitochondria are located. The phase contrast image shows the thymocytes. (×4200 zoom; Bar 5 μm).

regulation of non-shivering thermogenesis in mammals [1–3], its role in thymus is still a matter of investigation.

The objective of this investigation was to build upon our aforementioned empirical evidence for the existence of UCP 1 in thymocytes and to provide a definitive visual image of UCP 1 in thymocytes.

2. Materials and methods

2.1. Animal source

UCP 1 knock-out mice on a C57BL/6J background, originally provided by Dr. Leslie Kozak (Pennington Biomedical Research Center, Baton Rouge, Louisiana, US) were bred in-house (BioResources Unit, School of Biochemistry and Immunology, Trinity College Dublin.). All mice were housed in a specific pathogen-free facility and fed ad libitum. Both male or female mice were used and they weighed between 20 and 25 g.

2.2. Thymocyte isolation

Thymocytes were isolated from UCP-1 knock-out/wild type C57BL/6 mice essentially as described by Buttgereit and Brand [12]. The thymus was removed from the mouse, trimmed clean of connective tissue and brown fat (if present) and transferred into Hanks' Balanced Salt Solution (Sigma) containing 10% (w/v) foetal bovine serum (FBS). A single cell suspension was prepared by passage through a 70 μm nylon sieve (Falcon).

2.3. Antibodies and fluorescent probes

Rabbit anti-rat UCP1 IgG was obtained from Calbiochem. Mouse anti-rat Thy-1 was obtained from Abcam. CMXRos (Mitotracker Red), DNA stain (Hoechst 33342), Alexa 488 and Alexa 647-labelled secondary antibodies were obtained from Invitrogen.

2.4. Immunofluoresence

To visualize the mitochondria, CMXRos (Mitotracker Red)(200 nM final conc.) (Invitrogen) was added directly to the suspension medium for 60 min at 37 °C. Following this, 0.1% (v/v) paraformaldehyde was added to the thymocyte suspension and incubated on ice for 5 min. The thymocytes were allowed to dry completely on poly-L-lysine slides and were subsequently fixed with cold methanol (−20 °C) for 5 min and permeabilized with cold acetone (−20 °C) for 5 min. Finally, thymocytes were rinsed briefly with Phosphate Buffered Saline (PBS) buffer containing sodium azide (15 mM).

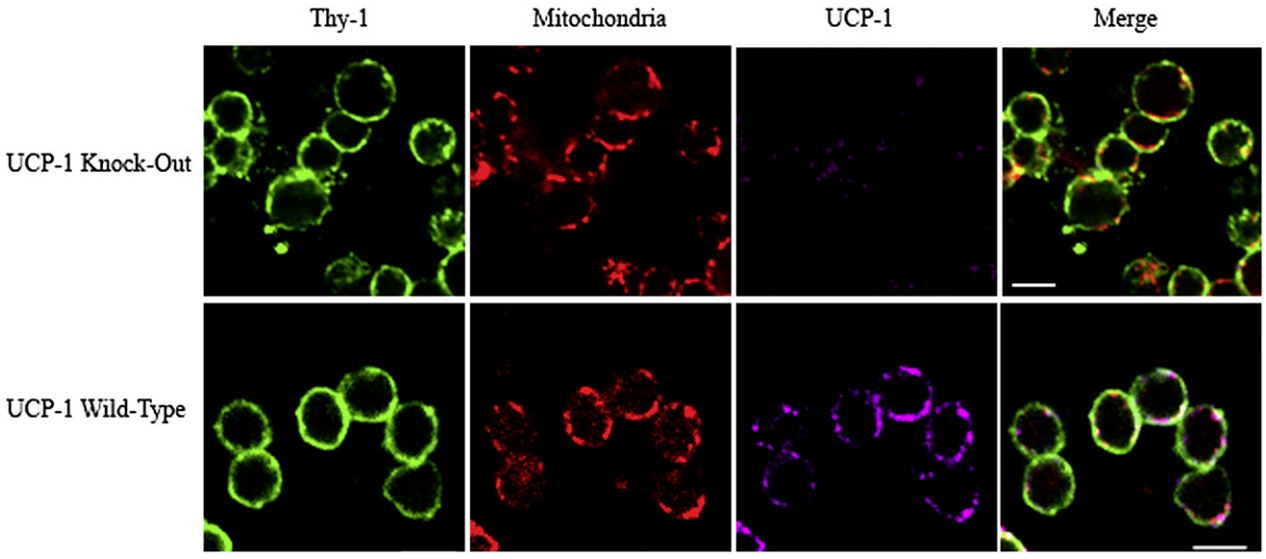

Fig. 2. In situ identification of UCP 1 in the mitochondria of thymocytes from UCP-1 wild-type mice, but not thymocytes from UCP-1 knock-out mice. Isolated thymocytes were incubated with Mitotracker Red for 60 min at 37 °C and fixed and permeabilized consecutively in methanol and acetone at −20 °C for 5 min each on poly-L-lysine slides and subsequently probed with the polyclonal anti-UCP 1 IgG and anti-Thy-1 (CD-90) IgG antibodies. UCP1 was detected using primary antibodies to UCP 1 and an Alexa 647 (magenta) labelled secondary antibody. Thy-1 was detected using an IgG primary antibody to Thy-1 and an Alexa 488 (green) labelled secondary antibody. Accumulated Mitotracker Red stains the mitochondria red. (×4200 zoom; Bar 5 μm).

Thymocytes were blocked with PBS containing 5% (w/v) bovine serum albumin (BSA) and 0.1 M methylamine for 1 h at room temperature. Following this, thymocytes were incubated with primary antibody in 5% (w/v) BSA overnight at room temperature, followed by incubation with the secondary antibody in 5%(w/v) BSA for 3 h at room temperature. After a brief rinse in PBS, the slides were mounted with coverslips and sealed. The mounting media used was 50% (v/v) glycerol in PBS containing 2% (w/v) *p*-phenylenediamine. The DNA stain, Hoechst 33342, was added to the mounting media at 1:100,000 dilution. Primary antibodies and dilutions used were as follows: rat anti-UCP-1 at 1:50, mouse anti-Thy-1 at 1:100. Alexa 488 and Alexa 647 were used at 1:1000 dilutions. Altogether, thymocyte preparations from 7 separate wild-type mice and 4 separate UCP 1 knock-mice were analyzed. Eight samples from each preparation were distributed into wells (all identical) and each well was analyzed over approximately 10 fields. The data presented are one of many examples of "best representative" data. Microscopy was performed on an Olympus FV1000 laser scanning Confocal microscope. A UPlanSAPO 60X/1.35 NA oil objective was used and the images were acquired with the accompanying software. Brightness and contrast adjustments and necessary cropping was performed using Photoshop Elements 5.0 (Adobe).

3. Results

Once thymi were cleared of all (any) surrounding tissue, thymocytes were isolated. Fig. 1 shows laser confocal microscopic images of thymocytes with fluorescent labelled surface antigen (Thy 1; CD-90; green), fluorescently labeled mitochondria (MitoTracker Red; red) and a merged image with the nucleus also stained (Hoescht stain; blue) plus light microscope images. It is interesting to note from the scale, that thymocytes are rather small cells (~5 μm in diameter) when compared to, for instance, brown adipocytes (~40 μm in diameter) [13] which are not present. The images show that the nucleus is clearly a prominent feature, taking up a large proportion of the thymocyte volume, with the mitochondria apparently located in a relatively smaller volume surrounding the nucleus, within the plasma membrane as determined by the Thy-1 antigen.

The visual evidence for the presence UCP 1 in thymocytes is clearly demonstrated in Fig. 2. We demonstrate that all cells investigated from UCP-1 wild-type mice and UCP-1 knock-out mice were thymocytes as determined by a monoclonal antibody specific for the thymocyte surface marker Thy 1 (CD90) pre-coupled to a fluorescent labelled (Alex 448, green). Mitochondrial location in thymocytes was determined using Mitotracker Red. Using a primary peptide antibody specific to UCP 1, and secondary fluorescently labelled (Alexa 647, magenta) antibody, we demonstrate that UCP 1 is associated with mitochondria in thymocytes from UCP 1 wild-type mice but not thymocytes from UCP1-knock-out mice. A merged image clearly demonstrates the relative location of the plasma membrane, mitochondria and UCP1 (in UCP 1 wild-type mice).

4. Conclusion

Our data provide unequivocal evidence for the presence of UCP 1 in thymocyte mitochondria in situ and are the first images to demonstrate UCP 1 expression in cells other than brown adipocytes. The demonstration by Frontini et al. [9] that UCP 1 is associated with BAT tissue in the vicinity of the thymus is not disputed, but their assertion that any UCP-1 associated with the thymus is due to BAT alone, is clearly not the case. We conclude and clearly demonstrate that mouse thymocytes contain UCP 1 in their mitochondria. Future work will determine the role of UCP-1 in thymus function.

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