The distinct bioenergetic properties of the human UCP1

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Abstract

The uncoupling protein UCP1 from brown adipose tissue is a mitochondrial carrier which allows dissipation of metabolic energy as heat. We have characterized the human UCP1 (HsUCP1) recombinantly expressed in *Saccharomyces cerevisiae* and we demonstrate that HsUCP1 is activated by fatty acids and retinoids in a nucleotide sensitive manner just as its rodent orthologs. However, in the absence of regulators, rodent UCP1 presents a high ohmic proton conductance that cannot be detected in HsUCP1. Since the human protein can be activated in a nucleotide sensitive manner, we conclude that it must have lost selectively the basal proton conductance.

Keywords: Uncoupling protein; UCP1; Brown adipose tissue; Bioenergetics; Fatty acid; Retinoid

Abbreviations: BAT, brown adipose tissue; FFA, free fatty acid; RCR, respiratory control ratio; TTNPB, 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid

1 Introduction

The uncoupling protein from brown adipose tissue (UCP1) is a mitochondrial transporter that allows a regulated discharge of the proton electrochemical potential gradient generated by the respiratory chain. This energy dissipatory mechanism has a thermogenic function. Brown adipose tissue (BAT) is used by mammals to maintain body temperature when cold exposed, and to burn off excess calories ingested to prevent obesity [1]. The uncoupling activity of UCP1 is regulated to meet with the energy dissipation requirements. The hypothalamus signals the initiation of thermogenesis through the sympathetic nervous system. Noradrenaline binding to β -receptors initiates a cAMP-dependent lipolytic cascade. The fatty acids released will become both substrates for oxidation and the second messengers that activate the proton conductance of UCP1 [2]. We have described that *all-trans* retinoic acid and other retinoids are also high-affinity activators of the protein [3]. Interestingly both, cAMP and retinoic acid, are also transcriptional activators of the *ucp1* gene [4].

We have previously reported that, when UCP1 is recombinantly expressed in the yeast *Saccharomyces cerevisiae*, the isolated mitochondria present bioenergetic properties very similar to mitochondria isolated from BAT [5]. Thus they display an enhanced basal membrane proton conductance that can be inhibited by purine nucleotides and a high sensitivity towards fatty acids and retinoids. UCP1 studies with *S. cerevisiae* mitochondria are greatly facilitated because there are no homologs of the uncoupling protein family in this unicellular organism. The comparisons between mitochondria from wild-type and UCP1-expressing yeasts resemble those done with mitochondria from wild-type and UCP1-KO animals [6] but circumvent the interference that may arise from the presence of other members of the UCP family.

The expression of UCP1 in *S. cerevisiae* has allowed an exhaustive investigation of its mechanism of transport and regulation, analyses of structure-function relationships, and the development of a high-throughput approach to search for new compounds that could be used to modulate its activity [7]. In our communication we present the recombinant expression of the human UCP1 in yeasts and its characterization. We compare its bioenergetic properties with those of two rodent UCP1 orthologs. We show that human UCP1 is also activated by fatty acids and retinoids but presents an intriguing alteration in its basal proton conductance.

2 Materials and methods

2.1 Construction of overexpression plasmids, yeast transformation and protein detection

Wild type UCP1 cDNA sequences from *Rattus norvegicus* (RnUCP1), *Mus musculus* (MmUCP1) and *Homo sapiens sapiens* (HsUCP1) were cloned in the pYEDP-1/8-10 vector under the control of the gal10-cyc1 promoter as previously reported [5]. The HsUCP1 was amplified by PCR from the pcMV6-XL5 plasmid (Origene, Rockville, MD). The modified pYEDP vectors were sequenced to confirm that the proper nucleotide sequence were present. Vectors were introduced into the *S. cerevisiae* strain W303 (a/α; ade2-1O; his3-11,15; leu2-3112; ura3-1; can1-100; trp-) by electroporation and transformed yeasts were selected for uracil autotrophy in SD minimal medium (0.67% yeast nitrogen base, 2% glucose, 0.1% casamino acids, 20 mg/l tryptophan, 40 mg/l adenine). Control yeasts were transformed with the same vector but containing the RnUCP1 sequence inserted in antisense orientation [5].

Protein expression and mitochondrial targeting were confirmed by Western blotting of mitochondrial preparations (Supplementary Fig. 1). The rodent proteins (RnUCP1 and MmUCP1) were detected using an antibody raised against the purified hamster UCP1 [5] while detection of HsUCP1 was performed using a commercial monoclonal human/mouse-UCP1 antibody (R&D Systems, Minneapolis, MN). The immunoblots were developed by enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK) and the band intensity recorded using the CCD camera of a Fujifilm LAS-3000 analyzer.

2.2 Yeast growth, isolation of mitochondria and bioenergetic characterization

Yeasts were routinely maintained in SD minimal medium. UCP1 expression was induced by growing yeast overnight at 28 °C in liquid SG medium (2% galactose, 0.67% yeast nitrogen base, 0.1% casamino acids, 20 mg/l tryptophan, 40 mg/l adenine) with vigorous shaking. Mitochondria were isolated by differential centrifugation after homogenization of spheroplasts as previously described [5]. UCP1 activity was evaluated from the effect of palmitate, *all-trans* retinoic acid and nucleotides (ADP and GDP) on mitochondrial respiration [3]. Respiration assays were performed on a Hansatech oxygen electrode. Assay medium contained 0.10–0.15 mg of mitochondrial protein per ml, 0.65 M mannitol, 2 mM MgCl₂, 10 mM phosphate, 0.5 mM EGTA, 10 μM fatty-acid-free albumin, 10 mM Tris-maleate pH 6.8. Substrate was 1 mM NADH. Protein concentration was determined by the BCA method using bovine serum albumin as standard.

2.3 Materials

Components for yeast growth media were from Difco Laboratories (Detroit, USA). Zymolase-20T was from Seikagaku Biobusiness Corp. (Tokyo, Japan). Nitrocellulose membranes were from Bio-Rad Laboratories. All other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

2.4 Statistical analysis

All values are expressed as mean ± SEM of the number of independent experiments indicated in the figure legends. All experiments were performed at least in duplicate. Differences between groups were determined using either two-tailed unpaired Student's *t*-test or the one-way ANOVA test using the SigmaPlot software. Significant differences between groups are indicated as *P < 0.01, and **P < 0.001.

3 Results

3.1 Human UCP1 does not present a basal proton conductance

It has been previously reported that UCP1 displays a proton conductance that can be observed in the absence of free fatty acids (FFA) or other activating ligands [8]. We have previously shown that recombinant expression of RnUCP1 in *Saccharomyces cerevisiae* introduces a proton leakage pathway in yeast mitochondria with bioenergetic and kinetic properties that resemble those found in isolated BAT mitochondria [5]. Thus, in isolated yeast mitochondria, the UCP1-dependent proton conductance can be recognized as a reduced respiratory control ratio (RCR) that is the result of an increased basal (state 4) respiration with a similar ADP-stimulated (state 3) rate (Supplementary Table 1). This higher state 4 rate can be lowered to levels similar to the control yeast mitochondria by addition of the inhibitory nucleotide GDP [3].

Fig. 1A shows the RCR values for the four yeast strains used in this study. Yeast mitochondria incorporating the rat or mouse UCP1 presented RCR values markedly lower than control yeast mitochondria while those containing HsUCP1 are not significantly different from control mitochondria. The analysis of the inhibition of GDP of the state 4 respiration clearly shows that there is a difference between the human UCP1 and the rodent orthologs. GDP inhibits the state 4 rate by 30% in the rodent UCP1 while no effect of the nucleotide is observed for HsUCP1 (Fig. 2B). GDP causes a slight, although statistically significant, increase in the state 4 rate, in control and HsUCP1 mitochondria that has been ascribed to the presence of an endogenous uncoupling pathway [9] probably linked to the yeast permeability transition pore [10]. Therefore, the lack of an effect of the incorporation of HsUCP1 in the state 4 rate would suggest that HsUCP1 does not present the basal proton conductance that is, however, present in rodent UCP1. The absence in HsUCP1 of the characteristic nucleotide sensitivity observed with the rodent UCP1 resembles the behaviour of the mutant Lp2_D of RnUCP1 where the substitution of two shared derived residues (Glu134 & Met140) by their counterparts in UCP2 (Asp and Phe respectively) abolishes the basal proton conductance [11]. However, these amino acid differences are not behind the distinct behaviour of HsUCP1 since the residues present in these positions are the same than in the rodent UCP1 (Supplementary Fig. 2).

Fig. 1 Bioenergetic properties of mitochondria isolated from control yeasts and strains expressing either rat UCP1 (RnUCP1), mouse UCP1 (MnUCP1), human UCP1 (HsUCP1) or the RnUCP1 mutant Lp2_D [10]. (A) Fold stimulation of respiration caused by the addition of 0.1 mM ADP (respiratory control ratio). (B) Inhibition of the basal rate of respiration caused by the addition of 1 mM GDP. Histograms show the mean value $\pm$ S.E.M. obtained from 8 to 10 (control & RnUCP1), 4 (MnUCP1) and 5 (HsUCP1) independent mitochondrial preparations in which the experiments were performed in duplicate. Statistically significant differences with respect to control mitochondria are indicated (* $P < 0.01$, ** $P < 0.001$).

alt-text: Fig. 1

Fig. 2 Stimulation of mitochondrial respiration by (A) palmitate or (B) the retinoid TTNPB in control mitochondria and mitochondria incorporating RnUCP1 or HsUCP1. The activators were added at the indicated effector:albumin molar ratios. Concentration of fatty-acid-free albumin in the incubation buffer was 10 μ M. Data points represent the mean value $\pm$ S.E.M. of 3-5 independent experiments performed at least in duplicate.

alt-text: Fig. 2

3.2 Human UCP1 is activated by fatty acids and retinoids

The absence of a basal proton conductance and the lack of a response to GDP could mean that the protein is inactive due, for example, to misfolding. Fig. 2 presents a comparison in the activation kinetics of HsUCP1 and RnUCP1 using two well-characterized activators of the proton conductance, palmitate and TTNPB [3,7], and shows that HsUCP1 containing mitochondria do respond to both activators in a GDP-sensitive manner while control mitochondria does not (Fig. 3). Since in the presence of palmitate 3:1 the rate of respiration for HsUCP1, mUCP1 and rUCP1 are not statistically different (see Supplementary Table 1), it can be assumed that the expression levels of the three recombinant proteins ought to be similar.

Fig. 3 Nucleotide sensitivity of the palmitate-induced activation of respiration in mitochondria from control yeasts and strains expressing either rat UCP1 (RnUCP1), mouse UCP1 (MnUCP1), human UCP1 (HsUCP1) or the RnUCP1 mutant Lp2_D. (A) Fold stimulation of respiration caused by the addition of 3:1 palmitate:albumin (molar ratio). (B) Inhibition of the palmitate-induced respiration caused by the addition of 1 mM GDP. Histograms show the mean value $\pm$ S.E.M. obtained from 3 to 5 independent mitochondrial preparation in which the experiments were performed at least in duplicate. Statistically significant differences with respect to control mitochondria are indicated (* $P < 0.01$, ** $P < 0.001$).

alt-text: Fig. 3

The fatty acid-induced proton conductance in the rodent and human UCP1 can be inhibited by GDP thus indicating that the lack of the nucleotide-inhibition of the basal conductance is not due to the loss of the nucleotide binding site (Fig. 3B). Again, the behaviour of HsUCP1 resembles the RnUCP1 mutant Lp2_D [11]. The two proteins retain the fatty acid sensitivity and the ability of purine nucleotides to inhibit the fatty acid-induced proton conductance despite the absence of a basal activity.

4 Discussion

In this report we have presented the bioenergetic characterization of the human UCP1 using a yeast expression system that has been previously shown to reproduce the biological activity of the protein in brown adipose tissue mitochondria [3,5,7]. This recombinant system has already been adapted to the requirements of a high throughput screening protocol thus opening the door to the search for new compounds that activate UCP1 [7] and that could then be used, for example, in obesity treatment. We demonstrate that HsUCP1 can be activated by retinoids and fatty acids and that this activation can be blocked by purine nucleotides just as the murine orthologs.

In the absence of purine nucleotides, UCP1 presents a high ohmic proton conductance that does not require the presence of activating ligands (fatty acids, retinoids, etc.) [7,12]. The existence of the basal conductance has generated controversy and it has often been attributed to the presence of endogenous FFA [13,14]. However, the earliest experiments on the bioenergetics of BAT mitochondria revealed that complete removal of FFA was not sufficient to observe energy coupling and that the presence of purine nucleotides was an absolute prerequisite [15-18 (Include here reference 19. Range would be [15-19])]. The presence of this basal conductance has been documented in brown fat mitochondria isolated from rat, hamsters, guinea-pigs, mice [8,20-22] and more recently from the tenrec (*Echinops telfairi*) a protoendothermic eutherian mammal [23]. This proton conductance observed *in vitro* is linearly correlated with the amount of protein present in the membrane [20]. Nevertheless, this conductance does not contribute to the mitochondrial proton leak *in vivo* since cytosolic purine nucleotides maintain the protein inhibited [14,24].

The expression of the human orthologue (HsUCP1) does not significantly lower the RCR and GDP has no effect on the basal rate of respiration. However, the protein is activated by fatty acids and retinoids in a nucleotide-sensitive manner hence HsUCP1 is active and its purine nucleotide binding site functional. These observations imply that HsUCP1 must have lost the nucleotide-sensitive basal proton conductance. This peculiar behaviour resembles the bioenergetic properties of the RnUCP1 mutant Lp2_D in which the residues Glu134 and Met140 were replaced by their counterparts in UCP2 (Asp and Phe respectively) [11]. UCP2 is a closely related mitochondrial carrier that does not present a basal proton conductance and whose transport activity is the exchange of malate, oxaloacetate and aspartate for phosphate [25]. Asp at position 134 is a highly conserved residue in the mitochondrial carrier family, being part of their signature sequence motif (PROSITE PS50920, PFAM PF00153) [26]. It is also worth noting that in the carp UCP1 position 134 is also Asp and its biochemical characterization did not reveal a nucleotide sensitive basal proton conductance while it was stimulated by fatty acids in a nucleotide sensitive manner [27]. Position 140 is occupied by Phe in UCP2, UCP3 and fish UCP1, whereas in all other UCP1 orthologs it is either Met or Leu. Therefore, the loss of the basal conductance in HsUCP1 resembles the mutant Lp2_D but the residues involved are not the same. Comparative sequence analysis between HsUCP1 and its murine orthologs reveals an 80% sequence identity but the comparison does not point to any conspicuous substitution (see Supplementary Fig. 2). A full

investigation by site directed mutagenesis will be necessary to establish the molecular basis of these functional differences.

Disclosure

The authors declared no competing interests.

Uncited references

[19].

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biochi.2016.10.005>.

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Appendix A. Supplementary data

The following are the supplementary data related to this article:

[Multimedia Component 1](#)

Supp. Fig. 1 Western blots for the expression of the UCP1 in control yeast mitochondria and in mitochondria isolated from yeasts expressing the different UCP1 orthologs. The primary antibodies used are indicated at the bottom of the blots. 10 µg of mitochondrial protein were loaded per lane.

alt-text: Supp. Fig. 1

[Multimedia Component 2](#)

Supp. Fig. 2 A, UCP1 sequence alignment for the mouse (MnUCP1), rat (RnUCP1), and human (HsUCP1) orthologs. B, Sequence alignment of the region corresponding to residues 123–175 in a set of protein sequences corresponding to the closest homologues to the human UCP1 (primates) and to the rat UCP1 (rodents). Positions 134 and 140 are highlighted.

alt-text: Supp. Fig. 2

Suppl. Table 1 Oxygen consumption rates of mitochondria isolated from control yeasts and strains expressing recombinantly different UCP1 orthologs.

alt-text: Suppl. Table 1

Highlights

- Activation of human UCP1 by fatty acids and retinoids is inhibited by purine nucleotides.
 - Rodent UCP1 orthologs present a nucleotide sensitive basal proton conductance.
 - Human UCP1 has selectively lost the basal proton conductance.
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