



# Not all mitochondrial carrier proteins support permeability transition pore formation: no involvement of uncoupling protein 1

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## Synopsis

The mPTP (mitochondrial permeability transition pore) is a non-specific channel that is formed in the mitochondrial inner membrane in response to several stimuli, including elevated levels of matrix calcium. The pore is proposed to be composed of the ANT (adenine nucleotide translocase), voltage-dependent anion channel and cyclophilin D. Knockout studies, however, have demonstrated that ANT is not essential for permeability transition, which has led to the proposal that other members of the mitochondrial carrier protein family may be able to play a similar function to ANT in pore formation. To investigate this possibility, we have studied the permeability transition properties of BAT (brown adipose tissue) mitochondria in which levels of the mitochondrial carrier protein, UCP1 (uncoupling protein 1), can exceed those of ANT. Using an improved spectroscopic assay, we have quantified mPTP formation in de-energized mitochondria from wild-type and *Ucp1*KO (*Ucp1*-knockout) mice and assessed the dependence of pore formation on UCP1. When correctly normalized for differences in mitochondrial morphology, we find that calcium-induced mPTP activity is the same in both types of mitochondria, with similar sensitivity to GDP (~50% inhibited), although the portion sensitive to cyclosporin A is higher in mitochondria lacking UCP1 (~80% inhibited, compared with ~60% in mitochondria containing UCP1). We conclude that UCP1 is not a component of the cyclosporin A-sensitive mPTP in BAT and that playing a role in mPTP formation is not a general characteristic of the mitochondrial carrier protein family but is, more likely, restricted to specific members including ANT.

**Key words:** adenine nucleotide translocase (ANT), brown adipose tissue (BAT), mitochondrial carrier protein, mitochondrial permeability transition pore (mPTP), mitochondrial swelling, uncoupling protein 1 (UCP1)

## INTRODUCTION

Mitochondria possess a Ca<sup>2+</sup>-inducible non-specific pore that allows the free movement of solutes of less than ~1.5 kDa across the mitochondrial inner membrane. Known as the mPTP (mitochondrial permeability transition pore), its activity is not compatible with the conventional chemiosmotic processes of mitochondria and causes matrix swelling, the collapse of protonmotive force and ATP depletion, leading to compromised cell viability. The mPTP has a clear involvement in necrotic cell death induced by calcium overload and oxidative stress, and may play a physiological role in the release of signalling factors from the mitochondrial intermembrane space during apoptosis (see [1–4] for reviews).

Although the components of the mPTP are yet to be conclusively identified, pore formation is proposed to involve cyclophilin D, ANT (adenine nucleotide translocase) and VDAC (voltage-dependent anion channel) [1,2]. Cyclophilin D is a mitochondrial-matrix-located peptidylprolyl *cis*–*trans* isomerase that associates with the mitochondrial inner membrane during conditions that promote permeability transition [5,6]. It is believed to induce a conformational change in a membrane protein to bring about pore formation [7]. The drug cyclosporin A inhibits cyclophilin D and is the classic inhibitor of the permeability transition [8,9]. Mice lacking cyclophilin D do not undergo cyclosporin A-sensitive permeability transition but still form mPTP at higher matrix Ca<sup>2+</sup> concentrations [10–12], indicating that cyclophilin D is not an essential component but may act to sensitize the Ca<sup>2+</sup> trigger site of the pore.

**Abbreviations used:** ANT, adenine nucleotide translocase; BAT, brown adipose tissue; mPTP, mitochondrial permeability transition pore; UCP1, uncoupling protein 1; *Ucp1*KO, *Ucp1*-knockout; VDAC, voltage-dependent anion channel.

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ANT has long been implicated in mPTP (see [7]) and is believed to be the membrane target of cyclophilin D. Ligands of ANT that promote the 'c' (carboxyatractyloside) or 'm' conformation (bongkreic acid) stimulate or inhibit mPTP respectively. Matrix ADP binding to ANT also inhibits mPTP and may be key in the mechanism by which mPTP formation is inhibited by  $\Delta\psi$  [7,13]. Importantly, a direct interaction between cyclophilin D and ANT has been demonstrated using cyclophilin D affinity columns and co-immunoprecipitation [3,14,15]. Strong evidence that supports ANT as the primary component of mPTP is provided by reconstitution studies. In response to high  $\text{Ca}^{2+}$  concentrations, phospholipid membranes containing purified ANT generate a non-specific pore that may represent a basic mPTP structure [16,17]. Reconstitution of ANT as part of a cyclophilin D-ANT-VDAC complex, recovered from a cyclophilin D affinity column, also results in  $\text{Ca}^{2+}$ -inducible pore formation that, importantly, can be inhibited by cyclosporin A [14]. The exact influence of VDAC in mPTP formation is not clear although mitochondria from mice ablated of VDAC1 exhibit unchanged permeability transition properties, suggesting that this isoform, at least, plays only a minor role [18].

Mouse mitochondria lacking ANT still exhibit cyclosporin A-sensitive mPTP formation, indicating that ANT is not an essential component of mPTP [19]. A possible explanation of this finding is that other proteins, similar to ANT, can perform the same structural role. ANT is typically the most abundant member of a large family of mitochondrial carrier proteins that share general structural features [20]. Formation of cyclosporin A-sensitive mPTP may be a general feature of this family and the well-established influence of ANT merely a reflection of its abundance over other mitochondrial carriers. To address this possibility, we have studied the permeability transition properties of BAT (brown adipose tissue) mitochondria from mice kept at 21°C, which are atypical in that they contain a mitochondrial carrier protein, UCP1 (uncoupling protein 1), at concentrations comparable with ANT [21]. Using an improved spectroscopic assay of mitochondrial swelling, we have quantified  $\text{Ca}^{2+}$ -induced mPTP formation in BAT mitochondria isolated from both wild-type and *Ucp1KO* (*Ucp1*-knockout) mice. When correctly normalized for differences in the relationship between mitochondrial absorbance and matrix volume, we find that mPTP formation in *Ucp1KO* mitochondria is the same as in wild-type mitochondria, but the fraction sensitive to cyclosporin A is increased. These results indicate that UCP1 does not catalyse cyclosporin A-sensitive mPTP and ANT's role in mPTP is not a general characteristic of the mitochondrial carrier protein family.

## EXPERIMENTAL

### Animals

Male and female mice were housed at  $21 \pm 2^\circ\text{C}$ ,  $57 \pm 5\%$  humidity, 12 h/12 h light/dark cycle, with standard chow and water *ad libitum*, following the U.K. Home Office Guidelines for the Care

and Use of Laboratory Animals. *Ucp1KO* [22] and wild-type sibling paired mice were used at age 7–12 months. *Ucp1* ablation was confirmed by Western-blot analysis of the protein and PCR analysis of the genomic loci.

### Isolation of mitochondria

BAT mitochondria were isolated as described in [23] but with the omission of EGTA in the final resuspension medium. Mitochondrial protein was estimated by the biuret method.

### Mitochondrial swelling

BAT mitochondria (0.15 mg) were stirred in 1.5 ml of reaction medium [0.25 M mannitol, 1 mM nitrilotriacetic acid (tetraethyl ammonium salt), 20 mM Mops buffer and 10 mM Tris buffer, pH 7.2 at 25°C] and the absorbance at 520 nm was followed by using a PerkinElmer lambda 18 spectrophotometer. To fully de-energize mitochondria and equilibrate  $\text{Ca}^{2+}$  across membranes, a cocktail of 0.2  $\mu\text{M}$  rotenone, 0.2  $\mu\text{M}$  antimycin A and 2  $\mu\text{M}$  A23187 was added after mitochondrial addition, followed by 1.3  $\mu\text{M}$  FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone). Swelling was induced by the addition of calcium gluconate to give the free  $\text{Ca}^{2+}$  concentration required under buffered conditions. Free calcium concentrations in the reaction medium were determined by following Calcium Green-5N (Molecular Probes, Invitrogen) fluorescence calibrated to calcium standards in reaction medium lacking nitrilotriacetic acid. Alternatively, immediate swelling was achieved on addition of mitochondria to reaction medium in which mannitol was replaced by 0.25 M erythritol. Swelling associated with erythritol uptake, specifically, was estimated by subtracting equivalent values of  $A^{-1}$  obtained in the presence of mannitol medium. In all cases,  $A^{-1}$  data were fitted using exponentials to estimate the initial rates ( $r^2 \geq 0.99$ ).

### Phospholipid estimations

Mitochondrial lipids were extracted using chloroform and methanol, and the phospholipid phosphorus content was estimated as described in [24]. Values given (see main text) are means ( $\pm$ S.E.M.) from five separate mitochondrial preparations.

## RESULTS AND DISCUSSION

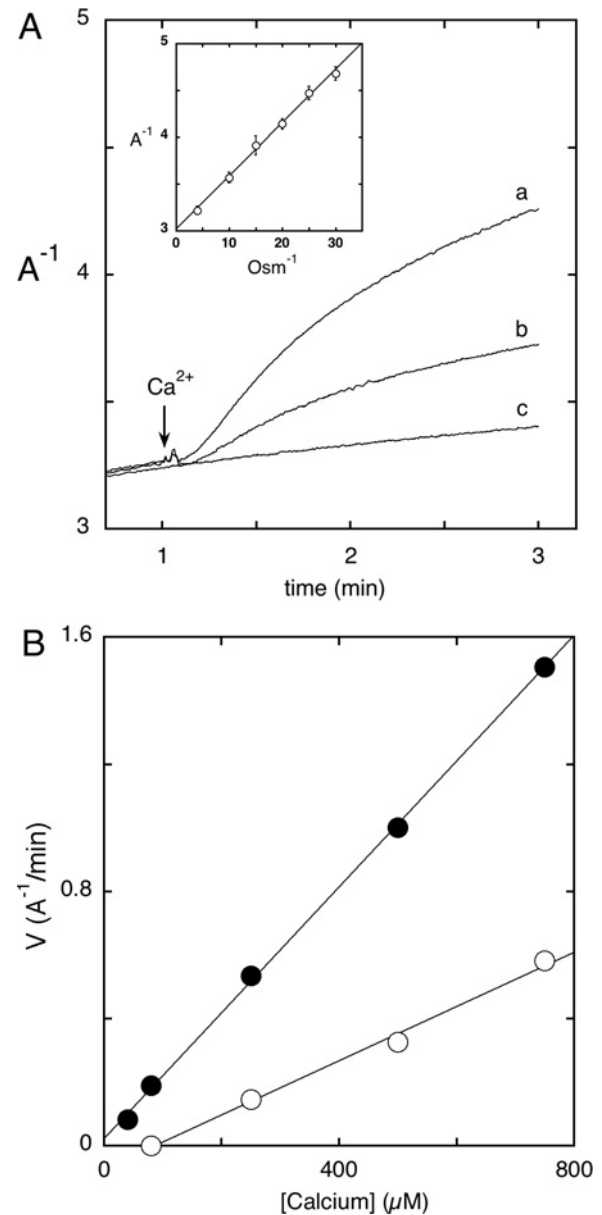
Time-resolved mPTP formation in isolated mitochondria has typically been assessed by monitoring the collapse of  $\Delta\psi$  or by following the absorbance changes associated with the matrix volume changes that accompany solute uptake. With either approach, the use of energized mitochondria complicates mechanistic interpretation as any affect on mPTP may be indirect, via  $\Delta\psi$ , particularly if UCP1 is involved. Also, neither approach is ideal for a quantitative analysis of the time course of mPTP formation. Mitochondrial absorbance measurements, for instance, suffer from a non-linear relationship with matrix volume that can vary

between mitochondria from different sources. To address this deficit in methodology, we developed an improved spectroscopic method to quantify cyclosporin A-sensitive mPTP in BAT mitochondria under de-energized conditions.

Mitochondria behave as simple osmometers in that their matrix volume is proportional to the inverse osmolality of the support medium [25–27]. In accordance with previous reports [26], we find that the inverse of the apparent absorbance of BAT mitochondria, equilibrated in media of various osmotic strengths, also changed linearly with inverse osmolality of the medium (Figure 1, inset). When expressed as  $A^{-1}$  and in the range measured, therefore, absorbance measurements can be used quantitatively to report relative changes in mitochondrial matrix water content. Addition of 500  $\mu\text{M}$  free  $\text{Ca}^{2+}$  (as calcium gluconate, the anion of which is not transported by UCPI [28]) to BAT mitochondria pre-equilibrated in 0.25 M mannitol medium induced rapid swelling associated with the uptake of mannitol (trace a, Figure 1A). This activity was inhibited by cyclosporin A (trace b), confirming that it is a consequence of mPTP formation. Intriguingly, inhibition occurred in the absence of phosphate in the incubation medium, in contrast with the situation in liver mitochondria where mPTP inhibition by cyclosporin A is dependent on the presence of this anion (see [29]). Phosphate regulation of mPTP inhibition by cyclosporin A, therefore, may not occur in BAT mitochondria, or instead, may just be dependent upon the assay conditions used. Importantly, in our assay conditions, mitochondria were kept fully de-energized (see the Experimental section) to avoid indirect effects of conventional UCPI activity on mPTP. Initial rates of  $\text{Ca}^{2+}$ -induced swelling were linearly dependent on the free  $\text{Ca}^{2+}$  concentration and were  $\sim 60\%$  sensitive to cyclosporin A (Figure 1B). This simple relationship suggests that our measurements do, indeed, report relative mPTP activities.

An initial assessment of mPTP formation in BAT mitochondria from *Ucp1KO* mice suggested that the rates of  $\text{Ca}^{2+}$ -induced swelling were lower than in mitochondria from wild-type mice (traces b and a respectively, Figure 2A). Caution must be taken, however, as these differences could have been due to general differences in the light scattering properties of *Ucp1KO* and wild-type mitochondria that were unrelated to mPTP. Indeed, a preliminary assessment of the dependence of  $A^{-1}$  on osmolality<sup>-1</sup> for *Ucp1KO* mitochondria indicated a linear, but shallower, relationship compared with wild-type mitochondria (results not shown). This suggests that the two mitochondrial populations were morphologically different and potentially exhibited different increments in  $A^{-1}$  for a specific amount of solute imported during permeability measurements.

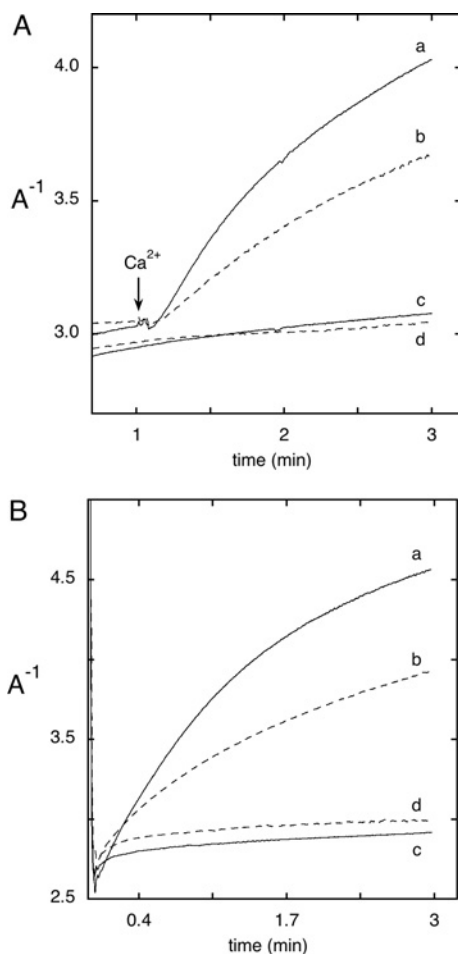
To correct for these differences, we measured swelling rates induced by erythritol. Biological membranes are permeable to this sugar, which enters mitochondria independently of mPTP and other transporters (see [30]). Rates of permeation per mg of mitochondrial protein will depend on membrane lipid composition and will be proportional to the membrane surface area. Wild-type and *Ucp1KO* mitochondria have the same phospholipid fatty acyl group composition [31] and our estimates of mitochondrial lipid phosphorus ( $2.7 \pm 0.2 \mu\text{g}/\text{mg}$  of protein for wild-type mitochondria and  $2.4 \pm 0.4 \mu\text{g}/\text{mg}$  of protein for *Ucp1KO* mitochon-



**Figure 1 Calcium-induced cyclosporin A-sensitive swelling of de-energized BAT mitochondria**

(A) Inverse of  $A_{520}$  (indicated by ' $A^{-1}$ ') associated with mitochondrial swelling induced by addition of 500  $\mu\text{M}$  free  $\text{Ca}^{2+}$  in the absence (a) or presence of 1.1  $\mu\text{M}$  cyclosporin A (b). The no calcium control is shown in (c). Inset: the dependence of  $A^{-1}$  associated with mitochondria, on inverse osmolality (indicated by ' $\text{Osm}^{-1}$ ') of the suspension medium. De-energized mitochondria were equilibrated for 1 min in reaction medium containing various concentrations of mannitol to give the required osmolality. Values are means ( $\pm$ S.E.M.) from four independent experiments. (B) The dependence of initial rates of calcium-induced swelling on the free  $\text{Ca}^{2+}$  concentration added in the absence (●) or presence (○) of 1.1  $\mu\text{M}$  cyclosporin A. Typical traces and rates are shown and are representative of at least one repeated experiment.

dria), as a marker of total lipid, suggested that they do not have significantly different surface areas. Therefore the initial rate of erythritol entry into both mitochondrial types should be the same, regardless of  $A^{-1}$  signal. Upon the addition of mitochondria to

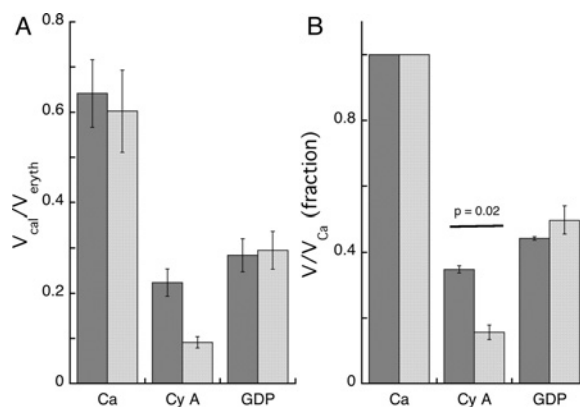


**Figure 2 Calcium- and erythritol-induced swelling of BAT mitochondria from wild-type and *Ucp1KO* mice**

Inverse of  $A_{520}$  ( $A^{-1}$ ) associated with the swelling of wild-type mitochondria (solid lines) and *Ucp1KO* mitochondria (dashed lines). (A) Swelling induced by 500  $\mu$ M free  $Ca^{2+}$  (a, b). No calcium controls (c, d). (B) Spontaneous swelling induced by addition of mitochondria to erythritol (a, b) or mannitol medium (c, d). Typical traces are shown and are representative of three repeated experiments.

0.25 M erythritol medium, it can be seen from Figure 2(B) that the spontaneous increase in  $A^{-1}$  associated with erythritol uptake in *Ucp1KO* mitochondria (trace b relative to trace d) was lower than in wild-type mitochondria (trace a relative to trace c). This confirms that the relationship between  $A^{-1}$  per mole of solute uptake was different in the two mitochondrial populations, which potentially accounts for the apparent decrease in the mPTP-dependent rates observed in *Ucp1KO* mitochondria (cf. Figure 2A). By expressing  $Ca^{2+}$ -induced rates as a function of erythritol-induced rates, mPTP activity in both types of mitochondria can be compared in the same units of solute influx.

Figure 3 shows the average rates of  $Ca^{2+}$ -induced swelling of wild-type and *Ucp1KO* mitochondria in the absence or presence of GDP or cyclosporin A. When correctly normalized to erythritol-induced rates, there was no effect of UCP1 ablation on  $Ca^{2+}$ -induced solute flux in the presence or absence of the UCP1



**Figure 3 Calcium-induced mPTP formation in BAT mitochondria from wild-type and *Ucp1KO* mice**

(A) Calcium-induced rates of swelling, obtained in the absence (Ca) or presence of 1.1  $\mu$ M cyclosporin A (Cy A) or 1 mM GDP, are shown normalized to erythritol-induced rates ( $V_{cal}/V_{eryth}$ ) for wild-type mitochondria (black bars) and *Ucp1KO* mitochondria (grey bars). Initial rates of mitochondrial swelling ( $A^{-1}/min$ ) were measured as described for Figure 2 (see the Experimental section). (B) Calcium-induced rates of swelling ( $V$ ) in the absence or presence of Cy A or GDP expressed as a fraction of the rate obtained with calcium alone ( $V_{Ca}$ ). All values are means ( $\pm$ S.E.M.) from three independent experiments. Values significantly different between wild-type and *Ucp1KO* mitochondria are indicated ( $P < 0.05$  by the paired Student's *t* test).

inhibitor GDP, suggesting that UCP1 is not involved in mPTP formation (Figure 3A). Note that UCP1-dependent mPTP formation in these results was not masked by compensatory changes in ANT concentration, as both mitochondrial types contain similar amounts of ANT as assayed by Western-blot analysis (results not shown). GDP inhibited mPTP formation by  $\sim 50\%$  (Figures 3A and 3B). This presumably occurs via an interaction with ANT, similar to the inhibitory effects of adenine nucleotides on mPTP [7], which is not without precedent as GDP has been shown to inhibit ANT-mediated proton conductance [32]. Intriguingly, the cyclosporin-sensitive fraction of mPTP activity was significantly higher in the absence of UCP1 ( $\sim 80\%$  inhibited by cyclosporin, compared with  $\sim 60\%$  in the presence of UCP1, Figures 3A and 3B). This could reflect a stress response that increases mPTP components, or their sensitivity to  $Ca^{2+}$ , indirectly related to the absence of UCP1 or it could reflect a minor role of UCP1 in cyclosporin A-insensitive mPTP formation. If either explanation were true, however, why a similar amplitude difference was not seen in the absence of cyclosporin A is not clear (Figure 3A). Alternatively, it may indicate changes in factors that influence cyclosporin efficacy. An increased concentration of endogenous phosphate associated with the loss of UCP1 could be one possibility, assuming that phosphate does indeed influence cyclosporin inhibition of mPTP in BAT mitochondria. Most importantly, these results show that  $Ca^{2+}$ -induced solute uptake activity, as well as the fraction of activity sensitive to cyclosporin A, was not lowered by UCP1 ablation, ruling out a structural role of UCP1 in the cyclosporin A-sensitive mPTP.

ANT is proposed to play a primary role in the formation of mPTP. However, Kokoszka et al. [19] have demonstrated that

mouse liver mitochondria in which ANT has been ablated still undergo cyclosporin A-sensitive permeability transition, albeit requiring higher calcium loads to do so. One interpretation of this finding is that other less abundant mitochondrial carrier proteins contribute to mPTP formation in the absence of ANT, reflecting a general role of this protein family in mPTP formation (e.g. [33]). In the present study, we developed a simple quantitative assay to study mPTP formation in mitochondria from wild-type and *Ucp1*KO mice. Our results demonstrate that UCPI does not facilitate the cyclosporin A-sensitive mPTP, indicating that a role in mPTP formation is not a general characteristic of the mitochondrial carrier protein family. There is strong evidence that ANT is a functional component of the mPTP, rather than just influencing pore activity. In particular, several studies have shown that mPTP-like activity can be achieved following reconstitution of purified ANT into lipid bilayers [14,16,17]. In view of our conclusions, a likely explanation of the finding of Kokoszka et al. [19] is that, in addition to ANT, only specific members of the mitochondrial carrier family are involved in mPTP formation. This may be restricted to carriers that are more widespread across various tissues. A particularly strong candidate is the mitochondrial phosphate carrier. This carrier has recently been shown to bind cyclophilin D, similarly to ANT, in affinity-column and co-immunoprecipitation studies and may, therefore, contribute to cyclosporin A-sensitive mPTP in the absence of ANT [34,35].

In summary, we have developed a simple quantitative assay of mPTP formation in BAT mitochondria and demonstrated that UCPI does not partake in cyclosporin A-sensitive mPTP formation. This finding is of significant importance as it eliminates a popular hypothesis that a role in cyclosporin A-sensitive mPTP formation is a general feature of the mitochondrial carrier protein family.

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