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Uncoupling protein-1 (UCP1) contributes to the basal proton conductance of brown adipose tissue mitochondria

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Abstract Proton leak pathways uncouple substrate oxidation from ATP synthesis in mitochondria. These pathways are classified as basal (not regulated) or inducible (activated and inhibited). Previously it was found that over half of the basal proton conductance of muscle mitochondria was catalyzed by the adenine nucleotide translocase (ANT), an abundant mitochondrial anion carrier protein. To determine whether ANT is the unique protein catalyst, or one of many proteins that catalyze basal proton conductance, we measured proton leak kinetics in mitochondria isolated from brown adipose tissue (BAT). BAT can express another mitochondrial anion carrier, UCP1, at concentrations similar to ANT. Basal proton conductance was measured under conditions where UCP1 and ANT were catalytically inactive and was found to be lower in mitochondria from UCP1 knockout mice compared to wild-type. Ablation of another abundant inner membrane protein, nicotinamide nucleotide transhydrogenase, had no effect on proton leak kinetics in mitochondria from liver, kidney or muscle, showing that basal proton conductance is not catalyzed by all membrane proteins. We identify UCP1 as a second protein propagating basal proton leak, lending support to the hypothesis that basal leak pathways are perpetrated by members of the mitochondrial anion carrier family but not by other mitochondrial inner membrane proteins.

Keywords Uncoupling protein (UCP) · Adenine nucleotide translocase (ANT) · Nicotinamide nucleotide transhydrogenase (NNT) · Proton leak · Carboxyatractylate (CAT) · Brown adipose tissue (BAT)

Abbreviations

ANT	Adenine nucleotide translocase
BAT	Brown adipose tissue
BSA	Bovine serum albumin
FCCP	Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone
KO	Knockout
NNT	Nicotinamide nucleotide transhydrogenase
TPMP	Triphenylmethylphosphonium
UCP	Uncoupling protein
WT	Wild-type

Introduction

During oxidative phosphorylation, substrates are oxidized by the mitochondrial electron transport chain, resulting in the pumping of protons out of the mitochondrial matrix. This

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proton pumping establishes a protonmotive force across the inner membrane, which is used by the F_1F_O-ATP synthase to synthesize ATP. There is, however, a futile cycle of proton pumping and proton leak (Brand et al. 1994; Nicholls et al. 1984) that may account for up to 25% of energy metabolism in a resting organism (Rolfe and Brand 1996, 1997). Proton leak pathways can be divided into two categories: inducible and basal. By definition, inducible leak can be activated or inhibited. Inducible pathways have been extensively investigated (Beck et al. 2007; Brand and Esteves 2005; Esteves and Brand 2005; Gonzalez-Barroso et al. 1998). Inducible leak is catalyzed by mitochondrial inner membrane carrier proteins, notably the uncoupling proteins (UCP1, UCP2 and UCP3) (Echtay et al. 2002; Esteves and Brand 2005) and the adenine nucleotide translocase (ANT) (Andreyev et al. 1989; Echtay et al. 2003; Parker et al. 2008a). Activators of inducible proton leak pathways include small molecules such as fatty acids (Andreyev et al. 1989; Esteves et al. 2006) or reactive oxygen species and their peroxidation products (Echtay et al. 2002, 2003; Parker et al. 2008b). Inducible proton leak is inhibited by the putative endogenous substrates of the mitochondrial carriers (Samartsev et al. 1997), purine nucleotides (Christiansen. 1971; Parker et al. 2008a), carboxyatractylate or bongkrekate (Azzu et al. 2008; Parker et al. 2008a).

Basal proton conductance is defined as the leak of protons into the mitochondrial matrix that occurs even when regulatable uncoupling pathways are inhibited. Genetic manipulation of ANT concentration in muscle mitochondria isolated from mice and fruit flies greatly altered basal proton conductance even when the carrier was fully inhibited with carboxyatractylate (Brand et al. 2005), showing that the ANT is the major contributor to basal proton conductance in these mitochondria. There are two interpretations of this result. Either ANT is the unique inner membrane protein responsible for basal proton conductance or other proteins are involved in this reaction and the major contribution of ANT is simply a result of its abundance (1–10% of total protein mass in mitochondria from all tissues tested). Of other uncoupling proteins, it is unsurprising that ablation of UCP2 and UCP3 in mice does not lead to a decrease in basal metabolism (Gong et al. 2000; Vidal-Puig et al. 2000; Zhang et al. 2001) or lowered basal proton conductance in mitochondria (Arsenijevic et al. 2000; Cadenas et al. 2002), since they only contribute 0.03% and 0.01% of total mitochondrial protein respectively (Azzu et al. 2008; Harper et al. 2002) and are not ubiquitous. By comparison, in wild-type mice maintained at room temperature (21–23 °C), UCP1 is present in brown adipose tissue (BAT) mitochondria at concentrations similar to ANT (Stuart et al. 2001), providing a rare opportunity to test the basal proton conductance activity of a second member of the mitochondrial anion transport protein family.

In this paper we examine UCP1 as a candidate mitochondrial inner membrane carrier protein involved in basal proton conductance under conditions of limiting endogenous fatty acids and full catalytic inhibition of both UCP1 and ANT. We find that BAT mitochondria from UCP1 knockout mice have significantly lower basal proton conductance than wild-type controls; UCP1 catalyzes about 75% of basal proton conductance in these mitochondria. We also show that another abundant, non-anion carrier inner membrane protein, nicotinamide nucleotide transhydrogenase (NNT), does not catalyze basal proton conductance in mitochondria from liver, kidney and muscle, implying that basal proton leak may be a unique property of the mitochondrial inner membrane carrier protein family.

Materials and methods

Animals

Male and female mice were housed at 21±2 °C, 57±5% humidity, 12/12 h light/dark, with standard chow and water ad libitum, following UK Home Office Guidelines for the Care and Use of Laboratory Animals. $Ucp1^{+/-}$ mice were used to derive $Ucp1^{-/-}$ (termed *Ucp1*KO) (Enerback et al. 1997) and wild-type, sibling paired mice. $Nnt^{+/-}$ (derived from a naturally occurring mutation in the C57Bl/6 line (Toye et al. 2005) crossed onto a wild-type 129 Sv strain) mice were used to derive $Nnt^{-/-}$ (termed *Nnt*KO) and wild-type sibling paired mice. Experimental animals were used at age 7–12 months. *Ucp1* and *Nnt* ablation was confirmed by PCR analysis of the respective genomic loci.

Isolation of mitochondria

Animals were killed by stunning followed by cervical dislocation and tissue was collected. All subsequent steps were carried out at 4 °C.

Liver, kidney and BAT mitochondria were isolated essentially as described (Echtay et al. 2002; Esteves et al. 2006). Tissue was placed in STE buffer (250 mM sucrose, 5 mM Tris/HCl, 2 mM EGTA, pH 7.4; supplemented with 1% (*w*/*v*) defatted BSA for BAT), minced and homogenized with a Dounce homogenizer. Homogenates were filtered through two layers of gauze and centrifuged at 8,500 g (10 min). The resulting pellets were resuspended in STE (+ BSA for BAT) and centrifuged at 700 g (10 min). The supernatant was recovered and centrifuged at 8,500 g (10 min). The pellet was suspended in STE, re-centrifuged and finally resuspended in STE.

Skeletal muscle mitochondria were isolated as described (Cadenas et al. 2002). Tissue was collected in CP1 buffer

(100 mM KCl, 50 mM Tris/HCl, 2 mM EGTA, pH 7.4) and minced with a sharp blade, then incubated for 3 min in CP1 supplemented with 1 mM ATP, 5 mM $MgCl_2$, 0.5% (*w*/*v*) defatted BSA, and 2.5 U/ml protease (type VIII). Homogenates were centrifuged at 490 g (10 min), and the supernatant was filtered through two layers of gauze then centrifuged at 10,400 g (10 min). The pellet was resuspended in CP1, centrifuged at 10,400 g (10 min), resuspended and centrifuged at 3,840 g (10 min). The final pellet was resuspended in CP1. Protein concentration was determined by the Biuret method.

Proton-leak kinetics

Proton leak rate across the mitochondrial inner membrane (in the presence of oligomycin to prevent ATP synthesis) was measured as the oxygen consumption required to translocate the leaked protons back out of the mitochondrial matrix. The kinetics of proton leak were measured as a function of the driving force, mitochondrial membrane potential (in the presence of nigericin to convert the pH component of protonmotive force to membrane potential) (Rolfe et al. 1994) and as such are independent of the substrate used to energize the mitochondria. Membrane potentials and respiration rates were measured simultaneously using electrodes sensitive to the membrane-potential dependent probe, $TPMP^+$ (triphenylmethylphosphonium), or oxygen. Mitochondria (0.35 mg of protein/ml) were incubated in assay medium containing (for hypotonic medium (Nicholls et al. 1972)) 50 mM KCl, 4 mM KH_2PO_4, 5 mM Hepes, 1 mM EGTA, 4% (*w*/*v*) defatted BSA (BAT) or, (for isotonic medium) 120 mM KCl, 5 mM KH_2PO_4, 3 mM Hepes, 1 mM EGTA 0.3% (*w*/*v*) defatted BSA (liver, kidney and skeletal muscle). Pilot studies showed that, although there was a decrease in proton leak between BAT mitochondria incubated with 3% BSA compared to 1% BSA, no further decrease was observed at 4% BSA, suggesting 4% BSA is sufficient to sequester endogenous free fatty acids. All assay media were supplemented with 5 μM rotenone, 1 μg/ml oligomycin, 0.11 μM nigericin and 2.5 μM carboxyatractylate and brought to pH 7.2. The $TPMP^+$ electrode was calibrated with sequential additions of $TPMP^+$ up to 2.5 μM and the mitochondria were energized with 10 mM glycerol-3-phosphate (BAT) or 4 mM succinate (other tissues). Membrane potential was decreased by sequential additions of the complex IV inhibitor KCN (14–230 μM) (BAT) or the competitive inhibitor malonate (0.1–2.3 mM) (other tissues). 0.2 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was added at the end of the run to release matrix $TPMP^+$ to allow for correction of any baseline drift. A correction of 0.4 $(\mu l/mg\ protein)^{-1}$ (liver) or 0.35 $(\mu l/mg\ protein)^{-1}$ (BAT and other tissues) was used to correct for TPMP binding; these values automatically correct for any changes in matrix volume (Brand 1995). Of note, the use of TPMP binding correction values quoted by Monemdjou et al. (1999) (0.20 and 0.25 $(\mu l/mg\ protein)^{-1}$ for wild-type and *Ucp1*KO respectively) affected neither the direction nor the statistical significance of differences in basal proton conductance between wild-type and *Ucp1*KO BAT mitochondria.

Western blot

Following isolation, mitochondria were solubilized in gel loading buffer (2% (*w*/*v*) SDS, 10% (*v*/*v*) glycerol, 1% (*v*/*v*) β-mercaptoethanol, 0.01% (*w*/*v*) bromophenol blue, 1 mM EDTA, 50 mM Tris-HCl, pH 6.8) and stored at −80 °C until use. Mitochondrial protein from thawed samples was separated on SDS/12% polyacrylamide gels, transferred to PVDF membranes (1 h at 100 V) and incubated overnight (4 °C) in blocking buffer (0.1% (*v*/*v*) Tween 20 and 5% (*w*/*v*) skimmed milk powder (Marvel) in phosphate-buffered saline). Membranes were probed with a rabbit anti-UCP1 polyclonal antibody (1:20 000 dilution; U6382, Sigma) or a goat anti-ANT polyclonal antibody (1:250 dilution; sc-9300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by the relevant peroxidase-conjugated secondary antibody (anti-rabbit at 1:20 000 dilution or anti-goat at 1:5,000 dilution; Pierce Biotechnology, Rockford, IL, USA). All antibody incubations were for 1 h at room temperature in blocking buffer. Antigens were visualized on Amersham Hyperfilm using an ECL Plus Western Blotting Detection system (Amersham Biosciences, Little Chalfont, Bucks., UK). For the quantification of ANT, band intensities were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/). For all blots, signal intensities were found to be linearly dependent upon the amount of mitochondrial protein loaded (5 to 20 μg per lane), confirming consistent loading and that the signal was not saturating.

Statistics

Respiration rates (by linear interpolation between flanking points) were calculated at the highest membrane potential common to all conditions on a given day. ANOVA was used to test for differences in these respiration rates.

Results

Pathways of proton conductance in brown adipose tissue

The mitochondrial carrier protein, UCP1, is known for its ability to catalyze inducible proton leak across the inner membrane of brown adipose tissue mitochondria in the presence of free fatty acids (FFA). We have investigated the ability of UCP1 to catalyze inducible and basal proton leak

with limiting endogenous FFA (4% (*w*/*v*) defatted BSA present) with ANT fully inhibited by carboxyatractylate (Fig. 1).

Figure 1a shows that, as expected, addition of a low concentration (160 μM) of palmitate (closed symbols) increased the proton conductance of wild-type BAT mitochondria containing UCP1 (squares), shifting the proton leak curve upwards such that measurement of proton leak kinetics remained possible. Addition of higher concentrations of palmitate led to near complete uncoupling of the BAT mitochondria. At 147 mV, the highest potential common to all experiments, 160 μM palmitate caused a significant increase in oxygen consumption rate driving proton leak (Fig. 1b), demonstrating that palmitate activates proton conductance in these mitochondria. Addition of 160 μM palmitate significantly induced proton conductance in BAT mitochondria containing UCP1, but had no effect on the proton conductance of BAT mitochondria in which UCP1 was ablated (Fig. 1a, triangles; Fig. 1b), demonstrating that palmitate was acting through UCP1 under these conditions.

Even in the absence of exogenous fatty acids, BAT mitochondria isolated from wild-type mice (Fig. 1a, open squares) still had significantly greater proton conductance than BAT mitochondria isolated from *Ucp1*KO mice (Fig. 1a, open triangles; Fig. 1b). This might represent residual UCP1 activity independent of fatty acids (Rial et al. 2004; Shabalina et al. 2004) or activity caused by contaminating fatty acids even in the presence of high BSA (Garlid et al. 1996). Alternatively, it may represent basal proton conductance caused by catalytically inactive UCP1. To distinguish residual inducible activity from basal proton conductance, we repeated the experiments in the presence of 1 mM GDP, previously demonstrated to fully inhibit fatty acid-activated UCP1 (Shabalina et al. 2004). GDP did not significantly recouple the BAT mitochondria from wild-type mice suggesting that high BSA was successful in preventing activation of the endogenous UCP1. Figure 1c demonstrates that, even after inhibition of UCP1, wild-type BAT mitochondria (light grey squares) had greater proton conductance than *Ucp1*KO mitochondria (light grey triangles) at membrane potentials above about 125 mV.

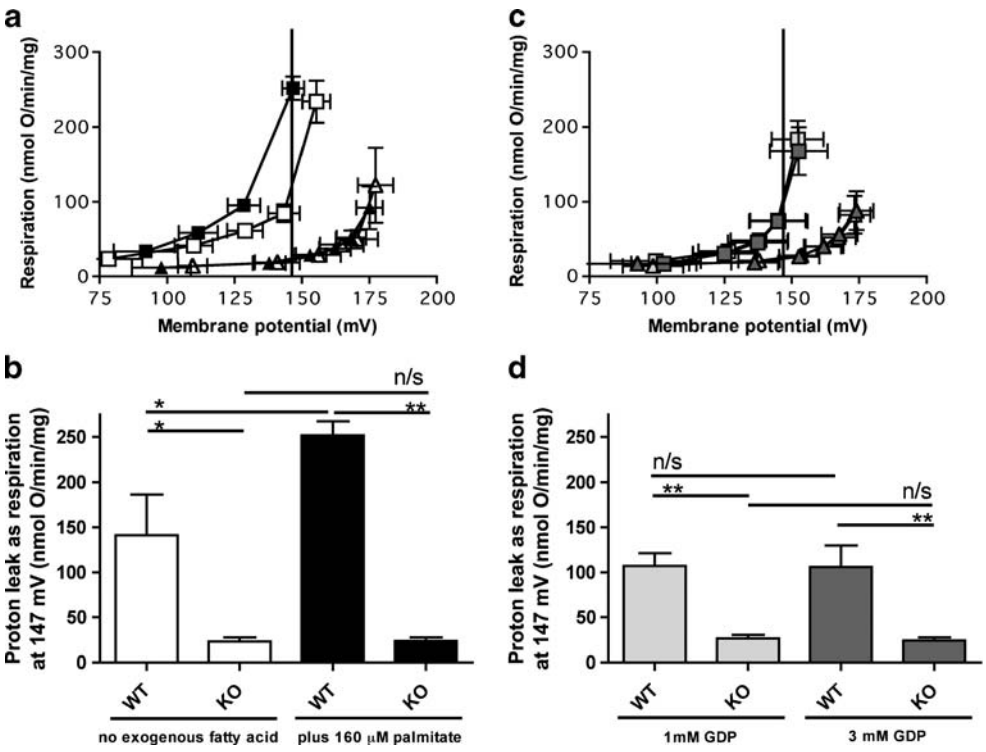

Fig. 1 Contribution of UCP1 to inducible and basal proton conductance in brown adipose tissue mitochondria. **a** and **c** show the kinetics of proton leak in BAT mitochondria isolated from wild-type (*squares*) and *Ucp1*KO mice (*triangles*) measured as described in Materials and Methods in the presence of 4% (*w*/*v*) defatted BSA and 2.5 μM CAT. The kinetic response of mitochondrial proton leak rate (measured as oxygen consumption rate) after energization with 10 mM glycerol-3-phosphate was measured (**a**) in the absence (*open symbols*) or presence (*closed symbols*) of 160 μM palmitate or (**c**) in the presence of 1 mM GDP (*light grey symbols*) or 3 mM GDP (*dark grey symbols*). **b** and **d** show the proton leak rate at 147 mV (*solid vertical lines* in A and C), the average highest membrane potential common to all curves; shading of bars corresponds to the shading of points in (**a**) and (**c**). Values are means ± S.E.M. of four experiments. One-way ANOVA was performed for **b**; $P=0.0001$, and **d**; $P=0.0014$, with Tukey's multiple comparison test used post-hoc; * $P<0.05$, ** $P<0.01$

Figure 1d shows respiration rates measured at 147 mV, the highest potential common to all experiments. The proton conductance of wild-type mitochondria containing catalytically inhibited UCP1 was significantly greater than the proton conductance of *Ucp1*KO mitochondria under the same conditions (light grey bars). To ensure that maximal inhibition of UCP1 had been achieved, proton leak kinetics were also measured in the presence of 3 mM GDP. Figure 1c and d show that there was no further inhibition of proton conductance at 3 mM GDP. These results show that UCP1 makes a major contribution (about 75%) to basal proton conductance in BAT mitochondria even when it is fully inhibited by GDP in the presence of high BSA and absence of exogenous fatty acids.

Regulation of ANT in *Ucp1*KO BAT mitochondria

The adenine nucleotide translocase contributes to both inducible (Andreyev et al. 1989; Samartsev et al. 1997) and basal (Brand et al. 2005) proton conductance. The proton leak kinetics shown in Fig. 1 were measured in the presence of the tight-binding inhibitor carboxyatractylate to prevent ANT-catalyzed inducible leak. However, any changes in ANT content caused by ablation of UCP1 would change the total contribution of ANT to basal proton conductance (which is insensitive to carboxyatractylate) and lead to an incorrect assessment of UCP1-mediated basal proton conductance. To assess the contribution of ANT content to differences in basal proton conductance, the amount of ANT was quantified.

Figure 2a shows that UCP1 was detected in mitochondria isolated from wild-type BAT but not in those from *Ucp1*KO BAT, but ANT was detected in both types of mitochondria. The ANT content in three separate paired wild-type and UCP1 knockout BAT mitochondrial preparations was measured by quantitative immunoassay. There was no difference (Fig. 2b), so secondary changes in ANT content do not explain the effects of UCP1 knockout on basal proton conductance.

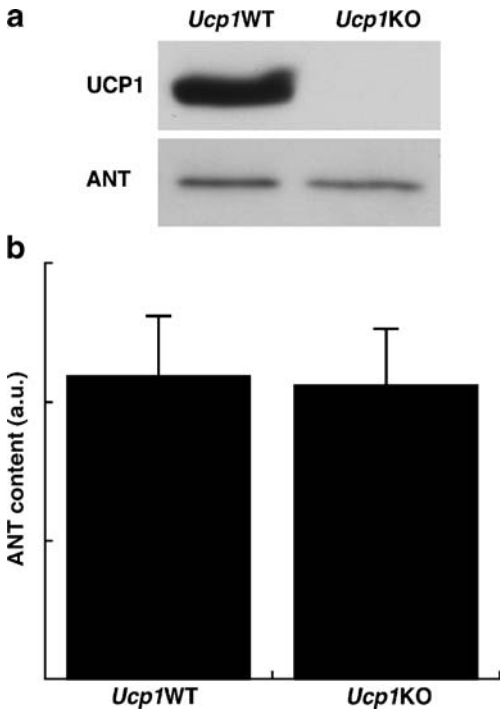

Fig. 2 ANT content in mitochondria from wild-type and *Ucp1*KO mice. **a**. Representative western blots of UCP1 and ANT in BAT mitochondria (10 μg/lane) isolated from wild-type and *Ucp1*KO mice. The UCP1 and ANT signals correspond to apparent molecular weights of ~32 and ~30 kDa, respectively. **b**. Densitometric quantification of ANT. ANT signal intensities were normalized to the total protein loaded into the wells, as determined by accurate protein measurement of mitochondrial preparations using the biuret assay. Pipetting errors in sample loading were minimised through repetition of the western transfer using the same mitochondrial preparation. Values shown, in arbitrary units (a.u.), are means ± S.E. (n=3), two blots per preparation on three separate, sibling paired mitochondrial preparations. ANT content was not significantly different between wild-type and *Ucp1*KO mitochondria (paired Student's t-test; p=0.16)

Contribution of transhydrogenase to basal proton conductance

The nicotinamide nucleotide transhydrogenase (NNT) constitutes 0.5–2% of total mitochondrial protein (Hojeberg and Rydstrom 1977; Wu et al. 1982), and was examined as an abundant representative of a different inner membrane protein family. Proton leak kinetics were measured in mitochondria isolated from wild-type and *Nnt*KO mice. The proton leak kinetics in mitochondria isolated from liver (Fig. 3a), kidney (Fig. 3b) or skeletal muscle (Fig. 3c) were similar when comparing wild-type (open symbols) with *Nnt*KO (closed symbols). Although there were the expected qualitative differences in proton conductance between different tissues (Rolfe et al. 1994), there were no significant differences in proton conductance with or without NNT (Fig. 3d). These data show that NNT does not catalyze basal proton conductance under the conditions tested.

Discussion

In this paper we examine the contribution of two different mitochondrial inner membrane proteins to basal proton conductance. It was previously shown that genetic modification of levels of ANT significantly alters basal proton conductance in isolated mitochondria (Brand et al. 2005). We show here that there was significantly reduced basal proton conductance in BAT mitochondria lacking UCP1 protein, in the presence of saturating levels of inhibitors of

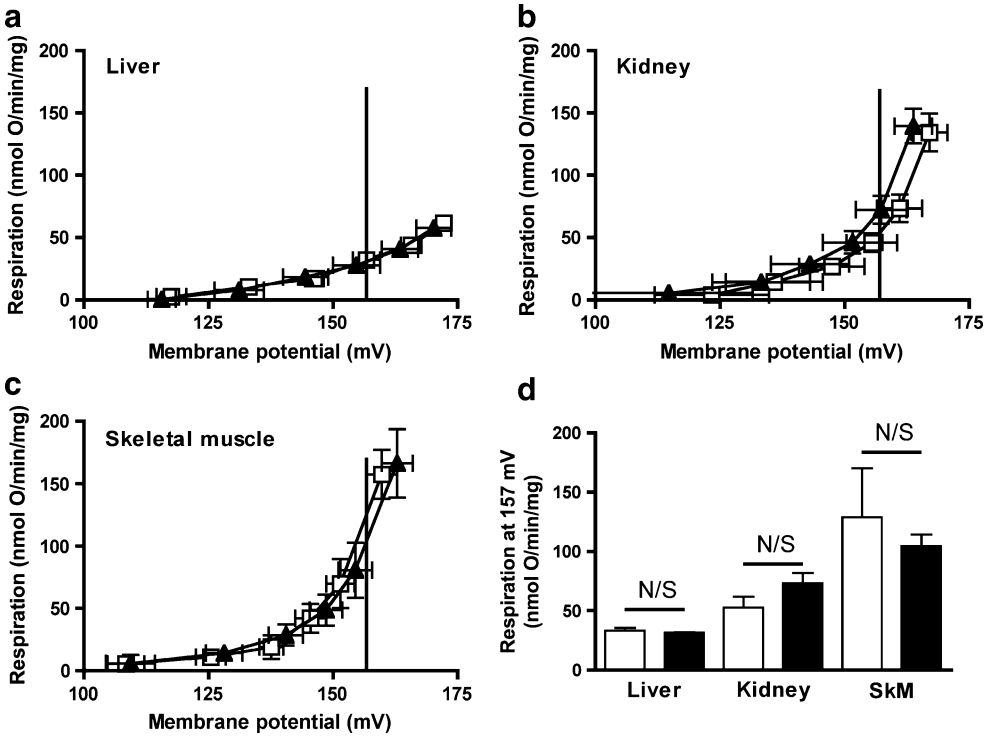

Fig. 3 Contribution of NNT to basal proton leak. **a**–**c** Proton leak kinetics were measured in mitochondria from wild-type (*open squares*) or *Nnt*KO mice (*closed triangles*) isolated from (**a**) liver, (**b**) kidney or (**c**) skeletal muscle (SkM) after energization with 4 mM succinate. Note all data are plotted on the same scale, with the horizontal axes truncated at 100 mV, making the curves for liver appear abnormally shallow. (**d**) Interpolation at 157 mV (the highest potential common to all experiments—solid vertical lines in A, B and C) allows direct comparison of proton leak rates (measured as oxygen consumption in the presence of oligomycin) between the different genotypes and tissues. Values are means ± S.E.M. of two (**a**), seven (**b**) and three (**c**) experiments. *N/S* indicates no significant difference between wild-type and *Nnt*KO samples (Student's t-test)

both ANT and UCP1 (CAT and GDP, respectively). However, mitochondria isolated from liver, skeletal muscle or kidney did not have decreased basal proton conductance in the absence of the abundant non-carrier protein, NNT. Although not exhaustive, these results suggest that basal proton conductance is a property shared by, and unique to, mitochondrial inner membrane anion carrier proteins.

Previous studies using mitochondria isolated from *Ucp1*KO or wild-type BAT suggested that UCP1 does not contribute to basal proton conductance (Monemdjou et al. 1999), however, these studies were performed in the absence of catalytic inhibition of ANT, so may have been compromised by inducible proton conductance through ANT, stimulated by an altered pool of endogenous fatty acids. Shabalina et al. (2004) found that UCP1 knockout did not affect non-phosphorylating oxygen consumption by BAT mitochondria respiring on pyruvate, and concluded that UCP1 has no role in basal proton conductance. However, non-phosphorylating, GDP-inhibited respiration with pyruvate as substrate is substantially lower than that seen when BAT mitochondria are respiring with glycerol-3-phosphate (Shabalina et al. 2004). The difference between these substrates can be explained by different patterns of control over respiration: with glycerol phosphate more control over resting respiration may lie in proton conductance, making it easier to observe effects of changes in proton conductance on respiration. If so, glycerol phosphate is a more appropriate substrate than pyruvate for indirect measurements of this type. Indeed, against the conclusion to their paper, Shabalina et al. (2004) show (without comment) that non-phosphorylating, state 4 respiration on glycerol-3-phosphate was decreased in UCP1 knockouts. Unfortunately this experiment was performed in the absence of CAT and without looking at the proton leak kinetics meaning it is not possible to discount the contribution of inducible proton leak due to ANT, or differences in mitochondrial membrane potential resulting in differences in state 4 respiration observed. Earlier experiments performed on BAT mitochondria isolated from warm (low UCP1) or cold (high UCP1) adapted guinea pigs (Locke et al. 1982; Rial and Nicholls 1984) or rats (Goubern et al. 1990) showed no apparent difference in basal proton conductance in the presence of GDP. In none of these studies were differences in fatty acid stimulated

leak through ANT accounted for. It is possible that the inducible proton conductance of ANT is higher in cold adapted animals due, for instance, to higher contaminating FFA in the mitochondrial preparation. Of interest, Goubern et al. (1990) showed differences in basal proton conductance between warm and cold adapted rats fed on an essential fatty acid deficient diet but these differences were interpreted as due to changes in fatty acid composition—they might actually have been due to differences in basal leak through UCP1. In our paper, we have looked at basal proton leak through UCP1 under more carefully controlled conditions. We minimized the effect of endogenous fatty acids on inducible proton conductance firstly by measuring proton leak kinetics in the presence of high concentrations of the fatty acid buffer BSA, and secondly through inhibition of known inducible leak pathways. Inhibition of ANT was particularly important because this FFA-activatable uncoupling protein has been reported to be upregulated in BAT mitochondria from UCP1 knockout mice (Shabalina et al. 2006), although we did not replicate this result (Fig. 2). Similar to the conclusion of Shabalina et al. (2004), work by Beck et al. (2006, 2007) suggested that there were no differences in current after a potential was applied to a planar lipid bilayer containing or not containing UCP1 protein. While this appears to be contrary to our findings, it should be highlighted that the planar lipid bilayer experiments were performed in artificial lipids that may not behave in the same way as physiological membranes. Additionally, the voltages applied to the planar lipid bilayers for the relevant conductance estimations were very low (<50 mV) compared to those measured in our experiments (~150 mV). The low membrane potentials used by Beck et al. (2006, 2007) may mask the membrane potential-dependent, non-ohmic basal proton conductance through UCP1 observed in our experiments. Indeed, this interpretation is supported by our kinetic analysis of proton leak (Fig. 1) where the curves, ± UCP1, become indistinguishable towards lower potentials.

Other studies have shown that, for example, surface area (Crichton et al. 2009) and phospholipid content (Ocloo et al. 2007) do not differ significantly between the wild-type and *Ucp1*KO models. This suggests that, while factors other than UCP1 content may influence basal proton conductance to some extent, they are unlikely to account for the large changes observed. We therefore conclude that a mitochondrial carrier other than ANT is able to promote basal proton leak. Of mitochondrial carriers, only ANT, the phosphate carrier and UCP1 (in BAT) constitute a sizeable share of mitochondrial inner membrane protein content and it is therefore difficult to assess basal proton conductance of other mitochondrial carrier protein family members. The lack of effect on basal proton conductance of ablation of each of the mitochondrial carrier family proteins in yeast (Roussel et al. 2002) may be due to their low individual abundance rather than their inability to catalyze basal proton conductance. In contrast, the absence of NNT (an inner membrane protein from a different family that is also present at significant concentrations) did not affect basal proton conductance so this is not an intrinsic property of all inner membrane proteins. NNT has been suggested to catalyze proton leak as a result of a futile cycle in which NNT uses protonmotive force to reduce NADP at the expense of NADH, and NAD- and NADP-isocitrate dehydrogenases re-equilibrate the two pools (Sazanov and Jackson 1994). We find no evidence that such a proton conductance pathway is significant under resting conditions in isolated mitochondria, because ablation of NNT does not alter basal proton conductance. In addition, significant futile proton cycling involving NNT should decrease weight gain in animals, but we found no differences in body mass between wild-type and *Nnt*KO animals (not shown). These data are inconsistent with NNT functioning in a significant energy-dissipating cycle either in isolated mitochondria or in vivo.

We conclude that basal proton conductance is catalyzed not only by ANT, as previously reported (Brand et al. 2005), but also by UCP1 in BAT mitochondria, where it is abundant. It is not catalyzed by NNT, an abundant membrane protein from a different protein family. By extension, we hypothesize that other members of the mitochondrial anion carrier family also contribute to basal proton conductance. Together, the basal proton conductance of many anion carriers at low concentration may account for the 34–50% of basal proton conductance not accounted for by ANT in muscle mitochondria (Brand et al. 2005).

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References

Andreyev A, Bondareva TO, Dedukhova VI, Mokhova EN, Skulachev VP, Tsofina LM, Volkov NI, Vygodina TV (1989) Eur J Biochem 182:585–592

Arsenijevic D, Onuma H, Pecqueur C, Raimbault S, Manning BS, Miroux B, Couplan E, Alves-Guerra MC, Goubern M, Surwit R, Bouillaud F, Richard D, Collins S, Ricquier D (2000) Nat Genet 26:435–439

Azzu V, Parker N, Brand MD (2008) Biochem J 413:323–332

Beck V, Jaburek M, Breen EP, Porter RK, Jezek P, Pohl EE (2006) Biochim Biophys Acta 1757:474–479

Beck V, Jaburek M, Demina T, Rupprecht A, Porter RK, Jezek P, Pohl EE (2007) FASEB J 21:1137–1144
Brand MD, Chien LF, Ainscow EK, Rolfe DFS, Porter RK (1994) Biochim Biophys Acta 1187:132–139
Brand MD (1995) In: Brown GC, Cooper CE (eds) Bioenergetics—A practical approach: measurement of mitochondrial protonmotive force. IRL, Oxford, pp 39–62
Brand MD, Esteves TC (2005) Cell Metab 2:85–93
Brand MD, Pakay JL, Ocloo A, Kokoszka J, Wallace DC, Brookes PS, Cornwall EJ (2005) Biochem J 392:353–362
Cadenas S, Echtay KS, Harper JA, Jekabsons MB, Buckingham JA, Grau E, Abuin A, Chapman H, Clapham JC, Brand MD (2002) J Biol Chem 277:2773–2778
Christiansen EN (1971) Eur J Biochem 19:276–282
Crichton PG, Parker N, Vidal-Puig AJ, Brand MD (2009) Biosci Rep. doi:10.1042/BSR20090063
Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA, Harper JA, Roebuck SJ, Morrison A, Pickering S, Clapham JC, Brand MD (2002) Nature 415:96–99
Echtay KS, Esteves TC, Pakay JL, Jekabsons MB, Lambert AJ, Portero-Otin M, Pamplona R, Vidal-Puig AJ, Wang S, Roebuck SJ, Brand MD (2003) EMBO J 22:4103–4110
Enerback S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, Kozak LP (1997) Nature 387:90–94
Esteves TC, Brand MD (2005) Biochim Biophys Acta 1709:35–44
Esteves TC, Parker N, Brand MD (2006) Biochem J 395:619–628
Garlid KD, Orosz DE, Modriansky M, Vassanelli S, Jezek P (1996) J Biol Chem 271:2615–2620
Gong DW, Monemdjou S, Gavrilova O, Leon LR, Marcus-Samuels B, Chou CJ, Everett C, Kozak LP, Li C, Deng C, Harper ME, Reitman ML (2000) J Biol Chem 275:16251–16257
Goubern M, Yazbeck J, Chapey MF, Diolez P, Moreau F (1990) Biochim Biophys Acta 1015:334–340
Gonzalez-Barroso MM, Fleury C, Bouillaud F, Nicholls DG, Rial E (1998) J Biol Chem 273:15528–15532
Harper JA, Stuart JA, Jekabsons MB, Roussel D, Brindle KM, Dickinson K, Jones RB, Brand MD (2002) Biochem J 361:49–56
Hojeberg B, Rydstrom J (1977) Biochem Biophys Res Commun 78:1183–1190
Locke RM, Rial E, Nicholls DG (1982) Eur J Biochem 129:381–387
Monemdjou S, Kozak LP, Harper ME (1999) Am J Physiol 276: E1073–E1082
Nicholls DG, Grav HJ, Lindberg O (1972) Eur J Biochem 31:526–533
Nicholls DG, Snelling R, Rial E (1984) Biochem Soc Trans 12:388–390
Ocloo A, Shabalina IG, Nedergaard J, Brand MD (2007) Am J Physiol Regul Integr Comp Physiol 293:R1086–R1093
Parker N, Affourtit C, Vidal-Puig A, Brand MD (2008a) Biochem J 412:131–139
Parker N, Vidal-Puig A, Brand MD (2008b) Biosci Rep 28:83–88
Rial E, Nicholls DG (1984) Biochem J 222:685–693
Rial E, Aguirregoitia E, Jimenez-Jimenez J, Ledesma A (2004) Biochim Biophys Acta 1608:122–130
Rolfe DFS, Brand MD (1996) Am J Physiol 271:C1380–C1389
Rolfe DFS, Brand MD (1997) Biosci Rep 17:9–16
Rolfe DFS, Hulbert AJ, Brand MD (1994) Biochim Biophys Acta 1188:405–416
Roussel D, Harding M, Runswick MJ, Walker JE, Brand MD (2002) J Bioenerg Biomembr 34:165–176
Samartsev VN, Mokhova EN, Skulachev VP (1997) FEBS Lett 412:179–182
Sazanov LA, Jackson JB (1994) FEBS Lett 344:109–116
Shabalina IG, Jacobsson A, Cannon B, Nedergaard J (2004) J Biol Chem 279:38236–38248
Shabalina IG, Kramarova TV, Nedergaard J, Cannon B (2006) Biochem J 399:405–414
Stuart JA, Harper JA, Brindle KM, Jekabsons MB, Brand MD (2001) Biochem J 356:779–789
Toye AA, Lippiat JD, Proks P, Shimomura K, Bentley L, Hugill A, Mijat V, Goldsworthy M, Moir L, Haynes A, Quarterman J, Freeman HC, Ashcroft FM, Cox RD (2005) Diabetologia 48:675–686
Vidal-Puig AJ, Grujic D, Zhang CY, Hagen T, Boss O, Ido Y, Szczepanik A, Wade J, Mootha V, Cortright R, Muoio DM, Lowell BB (2000) J Biol Chem 275:16258–16266
Wu LN, Pennington RM, Everett TD, Fisher RR (1982) J Biol Chem 257:4052–4055
Zhang CY, Baffy G, Perret P, Krauss S, Peroni O, Grujic D, Hagen T, Vidal-Puig AJ, Boss O, Kim YB, Zheng XX, Wheeler MB, Shulman GI, Chan CB, Lowell BB (2001) Cell 105:745–755