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Review

The reactions catalysed by the mitochondrial uncoupling proteins UCP2 and UCP3

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Abstract

The mitochondrial uncoupling proteins UCP2 and UCP3 may be important in attenuating mitochondrial production of reactive oxygen species, in insulin signalling (UCP2), and perhaps in thermogenesis and other processes. To understand their physiological roles, it is necessary to know what reactions they are able to catalyse. We critically examine the evidence for proton transport and anion transport by UCP2 and UCP3. There is good evidence that they increase mitochondrial proton conductance when activated by superoxide, reactive oxygen species derivatives such as hydroxynonenal, and other alkenals or their analogues. However, they do not catalyse proton leak in the absence of such acute activation. They can also catalyse export of fatty acid and other anions, although the relationship of anion transport to proton transport remains controversial.

Keywords: Uncoupling protein; Mild uncoupling; Superoxide; Reactive oxygen species; Reactive alkenal; Hydroxynonenal

1. Introduction

Since their discovery in the late 1990s, the 'new' mitochondrial uncoupling proteins UCP2 and UCP3 have been implicated in a wide range of physiological and pathological reactions, including thermogenesis, obesity, diabetes, ageing and degenerative, neurological, circulatory and immunological diseases. However, the interpretation of many of the experiments assumes that the UCPs catalyse a basal proton leak across the mitochondrial inner membrane that uncouples oxidative phosphorylation, without the need for other factors. This assumption appears to be incorrect. In this review, we consider what reactions are catalysed by UCP2 and UCP3. For more general background on the history and different viewpoints in the field, many reviews are available [1–11].

The new UCPs are closely related to UCP1, which catalyses adaptive thermogenesis in mammalian brown adipose tissue by greatly increasing the proton conductance of the mitochondrial inner membrane [12]. The leak of protons through the UCP1 proton conductance pathway uncouples substrate oxidation from phosphorylation of ADP to ATP, leading to fast oxygen consumption and heat production. The proton conductance of UCP1 is greatly enhanced by fatty acids, which may work by overcoming the inhibition by purine nucleotides such as ATP and GDP [13,14]. In the presence of physiological concentrations of nucleotides but the absence of fatty acids, UCP1 has no activity. Because of the sequence similarities between UCP1 and the newer UCPs, these properties of UCP1 might be shared by UCP2 and UCP3.

A speculative molecular structure of UCP1 based on the crystal structure of a member of the same family of mitochondrial carriers, the adenine nucleotide translocase, is shown in Fig. 1; the structures of the new UCPs that are discussed here are likely to be very similar. This UCP1 structure probably represents the general shape of

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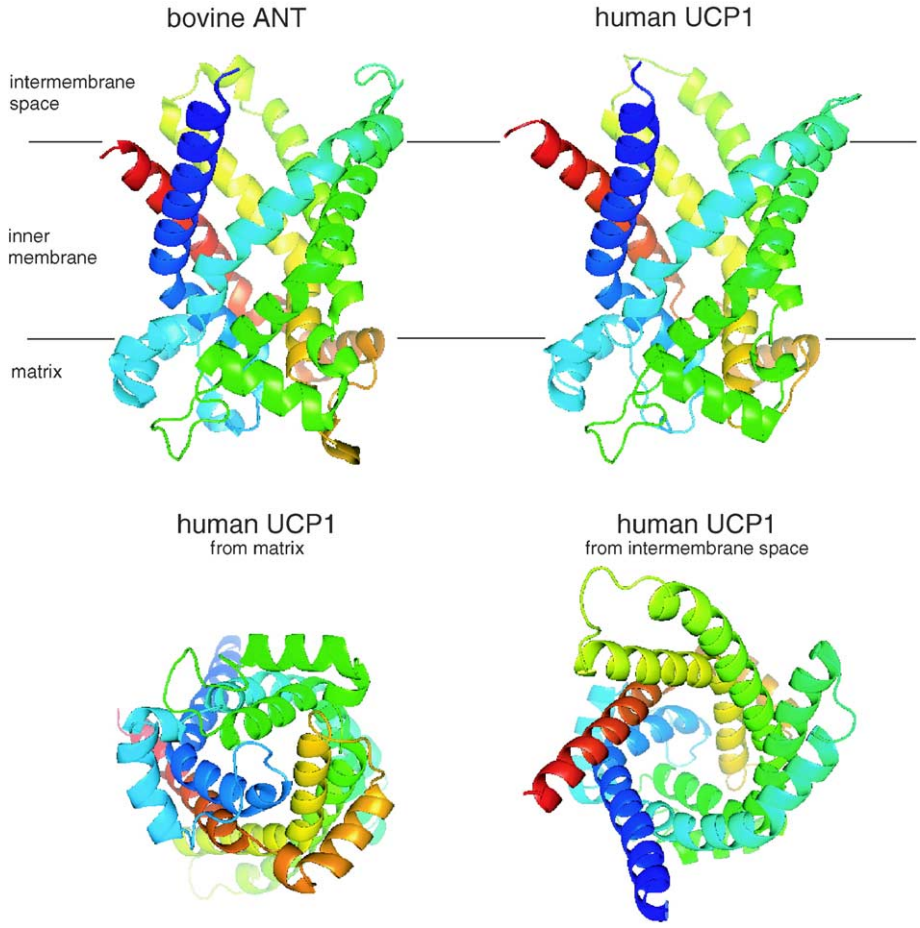

Fig. 1. Structure of the bovine adenine nucleotide translocase (ANT) and speculative structure of human UCP1. The carboxyatractylate-inhibited ANT structure was visualized in PyMol using published co-ordinates [94]. The sequence of UCP1 is folded into the structure of ANT to give the closest fit that avoids steric hindrance using the MODELLER comparative modelling program [95]. The UCP1 sequence fits the ANT structure very well, with only small differences in the connecting loops, suggesting that the model's major features are correct. Note that the crystal structure of ANT may not be identical to the native ANT structure, partly because of the bound carboxyatractylate, and any distortions in the ANT structure will also distort the derived UCP1 structure. UCP2, UCP3, avian and plant UCPs have close sequence similarity to UCP1 and are likely to have very similar three-dimensional structures, as are other members of the mitochondrial carrier protein family. (Courtesy of Alan Robinson, Dunn Human Nutrition Unit).

these proteins well, but it is not detailed or dynamic enough to allow insights into the reactions catalysed by the UCPs.

Whilst the thermogenic function of UCP1 is clear, the molecular details of how it transports protons and how it is regulated remain controversial. It is generally accepted that UCP1 catalyses two types of transport reaction across the membrane. The first reaction is net transport of protons (Figs. 2a, b, c, f). This might be direct (Fig. 2b), perhaps using the carboxylate of bound fatty acids as the prosthetic group [6,15], or indirect (Fig. 2c), by fatty acid anion export and flip back through the membrane of the protonated fatty acid [16–18]. The second reaction is export of chloride or fatty acid anions (Figs. 2c, d) [15]. The two reactions are either distinct or related, depending on the mechanistic model preferred. If proton transport is direct, then either the transported species is the hydroxide ion, or the anion transport pathway is a minor side reaction. If proton transport is indirect, the anion transport pathway is part of proton transport mechanism. Models for the reactions catalysed by UCP2 and UCP3 fall into the same two categories: either net proton transport or fatty acid anion transport as the primary reaction. As with UCP1, the two models may be related, but we shall discuss them separately.

Evidence for and against the biochemical and physiological functions of UCP2 and UCP3 has been widely discussed and critically reviewed [11,19]. In contrast to UCP1, they do not generally mediate adaptive thermogenesis, although UCP3 may be significantly thermogenic under specific pharmacological conditions [20]. There is strong evidence that the mild regulated uncoupling caused by UCP2 and UCP3 attenuates mitochondrial reactive oxygen species (ROS) production and protects against cellular damage [11,21,22], and (in the case of UCP2) diminishes insulin secretion [19]. It has also been suggested that UCPs play important physiological roles in the export of fatty acids [23,24] or of fatty acid hydroperoxides [25],

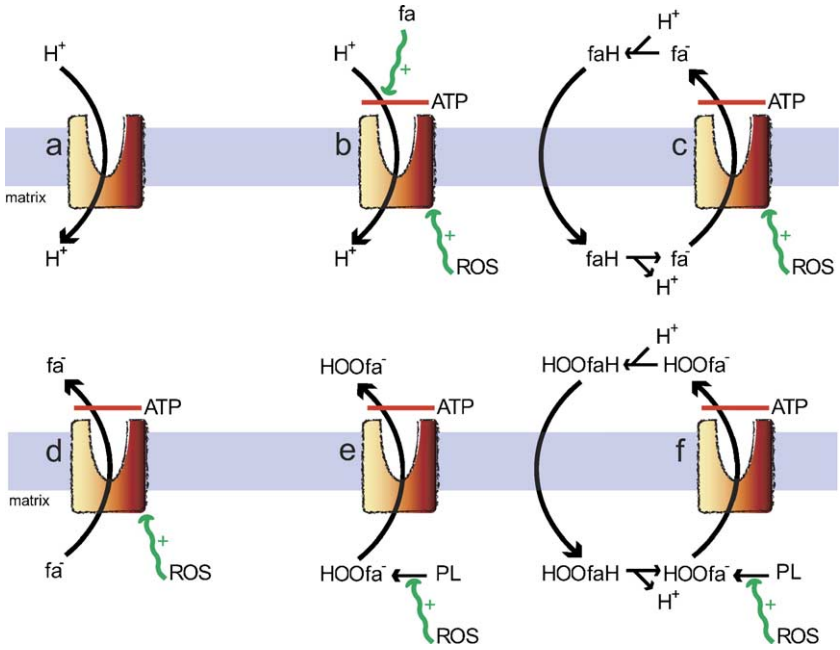

Fig. 2. Models for the reactions catalysed by UCP2 and UCP3 (and by avian and plant UCPs). Models a, b, c and f assume the UCPs catalyse net proton transport (Section 2); models c, d, e and f assume they catalyse fatty acid anion export (Section 3). (a) UCPs catalyse the basal proton conductance of the mitochondrial inner membrane; (b) UCPs catalyse an inducible proton conductance that can be activated by fatty acids (fa) and by derivatives of reactive oxygen species (ROS) (such as alkenals produced from superoxide, or analogues such as retinoate or TTNPB) and inhibited by purine nucleotides such as GDP or (physiologically) ATP; (c) as (b) but the net proton transport is catalysed by a cycle of fatty acid anion export followed by protonation and flip back of the uncharged protonated fatty acid (faH); (d) as (c) but faH flip back is quantitatively or functionally less important than the anion export; (e) as (d) but the species transported is the fatty acid hydroperoxide anion ($HOOfa^-$) produced by attack of ROS on membrane phospholipids (PL); (f) as (e) but the fatty acid hydroperoxide protonates and flips back to catalyse net proton conductance as in (c).

but evidence supporting these hypotheses is weak. UCP2 and UCP3 remain important potential targets for the treatment of ageing, degenerative diseases, diabetes and perhaps obesity.

2. UCPs increase the net proton conductance of the mitochondrial inner membrane

2.1. UCPs are activated by superoxide and alkenals

UCP2, UCP3, avian UCPs and plant UCPs transport protons and increase the net proton conductance of mitochondria, but only when specific activators are present (Figs. 2b, c, f) [21,22]. The proton conductance in the presence of each of these activators is inhibited by purine nucleotides such as ATP and GDP. Activators include ubiquinone [26,27], superoxide [26,28–34], AAPH (a carbon-centred radical generator) [29], reactive alkenals such as hydroxynonenal [35], and alkenal analogues such as cinnamate, retinal [35], retinoate and TTNPB [36,37]. The current working model of the interrelationship between these different activators [11,21,22] proposes that ubiquinone, superoxide and AAPH work indirectly, by generating carbon-centred radicals on polyunsaturated fatty acid chains of phospholipids in the mitochondrial inner membrane. These radicals are readily oxidised to lipid peroxyl radicals that autocatalytically release hydroxynonenal, which is proposed to be the direct activator of the UCPs. Cinnamate, retinoids and TTNPB are proposed to act as hydroxynonenal analogues, although retinoids and TTNPB may have additional effects due to their similarity to fatty acids.

Fatty acids or their analogues are required for activation of proton conductance by UCP2 or UCP3 in proteoliposomes [27,28,38] but not for alkenal activation in mitochondria [35]. It is possible that fatty acids activate UCP2 and UCP3 by relieving inhibition by nucleotides rather than activating directly, in the same way as proposed for UCP1 [13,14].

Fuller discussion of the evidence for and the interrelationships between these activators is presented elsewhere [11,21,22].

2.2. UCPs are not functional in the absence of activators

UCP2 and UCP3 catalyse an inducible proton conductance in the presence of activators, but they do not catalyse the basal proton conductance of mitochondria (i.e., the conductance that is measured in the absence of these activators, Fig. 2a). Reaching this understanding has been a gradual process for the whole field, and there are still a few dissenters. For example, some authors [39] have not observed the retinoate and superoxide activation of UCPs, and various artifacts that might compromise the conclusion that the new UCPs only transport protons when activated

have been suggested [39,40]. However, the criticisms have been refuted [30], and several groups have now reported activation of the proton conductance of UCP2 or UCP3 in mitochondria and intact cells [21,22,32,34,36]. In intact brown adipose tissue mitochondria, UCP1 is active when both fatty acids and nucleotides are removed [38]. However, in proteoliposomes, the absence of these effectors is not sufficient to allow activity, and ubiquinone (presumably to generate reactive alkenals [31]) and fatty acids (perhaps to displace residual nucleotides) are required for full activity [27]. The simplest interpretation of these observations is that UCP1, like UCP2 and UCP3, does not catalyse proton conductance unless it is activated by reactive alkenals, but endogenous alkenals already activate the protein in brown adipose tissue mitochondria in vitro.

Based on the new understanding that UCP2 and UCP3 only catalyse proton conductance when suitably activated, we can now revisit the recent literature and review published results.

2.3. Basal proton conductance—heterologous expression of UCPs in yeast

Yeast readily express mammalian proteins, and provide an established system for testing the functions of mitochondrial proteins. In early studies, mammalian UCP2 or UCP3 were expressed in yeast mitochondria. Indirect or direct measurements showed that they increased GDP-insensitive uncoupling in the absence of added activators (Fig. 2a), suggesting that they catalyse a native basal proton conductance [41–48]. Similar observations have been made with an avian UCP [49] and with the more distantly related protein, BMCP1 [50]. However, gain-of-function for a leak across a membrane can be problematic, since misfolded protein can give a false positive. In gain-of-function studies of this sort, it is vital that some secondary criterion, such as sensitivity to nucleotides, is employed. It subsequently became clear that both UCP2 [51] and UCP3 [52–54] are misfolded or poorly incorporated into the membrane when expressed in yeast mitochondria, and cause an artifactual, nucleotide-insensitive increase in basal leak that does not increase linearly with UCP concentration. Importantly, this leak is not stimulated by retinoids [51] or superoxide [54], so not only do the proteins artifactually increase proton conductance, but they also fail to carry out their native function.

Nevertheless, some UCPs do function in yeast. It is well established that mammalian UCP1, when not overexpressed too strongly in yeast mitochondria, catalyses activator-dependent, GDP-sensitive proton conductance (Figs. 2b, c) [31,43,44,54–58]. In one report, UCP2 in yeast was stimulated by retinoate, although it was not GDP-sensitive [36], suggesting that UCP2 may be partially functional in yeast in some circumstances. A recent report of fatty acid-inducible GDP-sensitive uncoupling in yeast expressing a *Drosophila* protein similar to mammalian BMCP1 [59] raises the otherwise unsubstantiated possibility that these proteins may also catalyse inducible proton conductance in yeast.

Thus experiments showing increased GDP-insensitive uncoupling of yeast mitochondria expressing mammalian UCP2 and UCP3 cannot be accepted as evidence that these proteins, when properly folded, catalyse a basal proton conductance. By extension, other studies showing uncoupling following expression of a mitochondrial membrane protein in yeast should be rejected unless they provide compelling evidence that the reaction is not an expression artifact.

2.4. Proton transport by UCPs in proteoliposomes

Incorporation of UCPs into proteoliposomes provides an appealing system for studying the reactions catalysed by the new UCPs. However, there are technical problems concerning the state and purity of the protein fractions used, the integrity of the protein and lipid molecules after proteoliposome formation, and the heterogeneity and usually the lack of characterization of membrane potentials and pH gradients. Several studies have shown that UCP2 and UCP3 can cause a net proton conductance. However, the crucial inhibition by nucleotides (as demonstrated in experiments using isolated mitochondria) has been problematic, with many studies finding very poor nucleotide sensitivity [60–62], raising the possibility of artifact as discussed above. Some studies find high sensitivity to nucleotides [27,63,64], and are therefore likely to be more reliable. Proton transport requires fatty acids (Figs. 2b, c) [27,60]. In some studies, it also requires addition of ubiquinone [27], which we interpret as a requirement for a source of radicals to initiate the alkenal activatory pathway [31]. Preparations in which ubiquinone is not required, or is only weakly activatory, [62,64] may be contaminated with endogenous ubiquinone or other radical generators, or subjected to oxidative stress.

Thus, experiments in proteoliposomes provide evidence that UCP2 and UCP3 can catalyse a nucleotide-sensitive proton conductance, but only in the presence of fatty acids and probably also free radical generators (Figs. 2b, c, f).

2.5. Does proton conductance correlate with natural alteration in UCP levels?

Although they are more complex and may lack factors that are important in vivo, isolated mammalian mitochondria naturally expressing different amounts of UCPs provide a well-understood and experimentally amenable system that avoids the disadvantage of potential expression artifacts.

There are examples where physiological changes in UCP2 or UCP3 mRNA or protein content correlate with thermogenesis, such as after lipopolysaccharide or cytokine administration [65,66], or with mitochondrial proton conductance, such as in liver mitochondria from ob/ob mice [67]. Importantly, there are examples where they do not:

fasting raises UCP3 mRNA and protein in rat skeletal muscle, but decreases thermogenesis [68] and does not alter the basal proton conductance of the muscle mitochondria [69,70].

The lack of consistent correlation between UCP2 or UCP3 content and basal proton conductance is a strong indication that these proteins do not catalyse the basal proton conductance of isolated mitochondria, although it is theoretically possible that changes in other pathways could swamp any effects of changes in UCP content on basal proton conductance. It should also be mentioned that changes in mRNA or even protein levels for UCPs cannot predict the functional effect (which depends on the activation state of the protein in situ), but can only predict the capacity of the protein for activation. Therefore, the functional relevance of studies of UCPs that measure only mRNA levels must be considered with caution.

2.6. Experiments using UCPs overexpressed in mammalian cells

UCPs have been artificially expressed in mammalian cells, which should allow a clearer understanding of the roles of UCPs in a relevant cellular context. The big disadvantage is that, without independent corroboration, such gain-of-function studies are potentially subject to the expression artifacts discussed above. UCP1 can be expressed in mammalian CHO cells to give GDP-sensitive mitochondrial uncoupling [71]. In contrast, the functions ascribed to UCP2 and UCP3 in such studies are almost never demonstrated to be due to any native function rather than to artifactual uncoupling.

There are many studies that are potentially interesting but inadequately controlled for artifactual uncoupling when UCPs are expressed in mammalian cells. For example, overexpression of UCP2 in pancreatic beta cells [72], cardiomyocytes [73] or pheochromocytoma cells [74] increases cell survival after hydrogen peroxide treatment; overexpression of UCP2 in macrophages reduces ROS and increases nitric oxide production [75]; overexpression of UCP3 in myotubes or cardiomyoblasts increases glucose uptake [76] and overexpression of UCP3 in myoblasts [77], UCP2 in HeLa cells [78] or UCP3 or UCP4 in kidney cells [79], reduces mitochondrial potential.

Some workers have tried to address the criticism of artifactual uncoupling. Mitochondria from pancreatic beta cells transfected with UCP2 show a dose-dependent increase in basal proton conductance, but this is GDP-insensitive [80]. The authors argue that this effect of UCP2 is specific, since uncoupling following UCP1 expression in the same system is GDP-sensitive, and expression of the oxoglutarate carrier does not uncouple. However, given the known GDP-sensitivity of inducible proton conductance through UCP2 and the clear differences between UCP1 and UCP2 in yeast (Section 2.3), this conclusion seems optimistic and the UCP2 effect is probably an overexpression artifact. Similarly, reports that expression of UCP2 or UCP3, but not the adenine nucleotide or oxoglutarate translocases, causes uncoupling [78,81] may well reflect expression artifacts rather than any native UCP function.

There is one clear demonstration that overexpression of UCP3 in mammalian cells can cause aberrant protein folding and artifactual uncoupling [82]. Mitochondria from myoblasts overexpressing UCP3 show a dose-dependent increase in respiration, but this is unaffected by fatty acids or GDP. The UCP3 is resistant to detergent extraction by methods that solubilize membrane proteins, suggesting that it is coagulated. Thus, UCP3 overexpressed in these mammalian cells results in increased mitochondrial uncoupling that does not represent a physiologically relevant function of the protein. By contrast, modest levels of functional UCP3 can be expressed in mammalian 293 cells [83]; the mitochondria show GDP-sensitive changes in respiration and membrane potential on addition of fatty acids.

Thus overexpression of UCP2 and UCP3 in mammalian cells sometimes leads to functional UCP (Figs. 2b, c, f), but sometimes causes only an uncoupling artifact (Fig. 2a). Without good evidence that the phenotype in any particular case is not caused by the expression artifact, such experiments cannot be accepted as solid evidence that these proteins, when properly folded, catalyse a basal proton conductance or would produce the observed phenotypes.

2.7. Transgenic overexpression of UCPs in animals

Mice transgenically overexpressing UCP2 or UCP3 have been examined by several groups. This approach should amplify the behaviour of UCPs in their physiological context. As above, the great disadvantage is that, without independent corroboration of native function, such gain-of-function studies are potentially explainable simply by uncoupling artifacts. To date, no study in which UCP2 or UCP3 has been overexpressed in vivo has shown convincingly that the effects seen are due to native UCP function rather than to artifactual uncoupling.

A clear demonstration that UCP3-mediated proton conductance in vivo can lead to an antiobesity phenotype is provided by mice that overexpress UCP3 in skeletal muscle. They are hyperphagic yet leaner than wild-type [84]. Their skeletal muscle mitochondria are less well coupled and have higher proton conductance than wild-type mitochondria, explaining the antiobesity effect. However, this phenotype is the result of an overexpression artifact [85]. The increase in proton conductance is not proportional to the increase in UCP3 protein, and, importantly, it is neither activated by superoxide nor inhibited by GDP, showing that the uncoupling and the antiobesity phenotype it causes result not from functional UCP3, but from an overexpression artifact (Fig. 2a).

Mice transformed with a UCP2/UCP3 construct have less fat [86,87] and increased ethanol tolerance [88]. Brain

damage after induced stroke is reduced, and hippocampus homogenates show uncoupling and lower radical-induced damage [74,89]. The authors conclude that UCP2 is physiologically neuroprotective, but the only hint that UCP2 may be functional is that brain mitochondria from the transgenic animals are more sensitive to uncoupling by fatty acids—neither GDP-sensitivity nor alkenal activation are reported [89].

Thus, the uncoupling seen in mitochondria from mice that are engineered to overexpress UCP2 or UCP3, and the phenotypes that result from this uncoupling, cannot be taken as solid evidence that these UCPs naturally catalyse the basal proton conductance of mitochondria. Instead, they provide useful insights into the phenotypes that mild (unregulated, artifactual) uncoupling can cause in different situations.

2.8. Transgenic animals underexpressing UCPs

The great advantage of loss-of-function experiments is that they are not subject to the artifacts potentially caused by overexpression of UCPs. Disadvantages include the possibility of compensatory expression of other genes that mask the effects of the loss of function.

The first studies using muscle mitochondria isolated from UCP3 knockout mice found evidence for decreased proton conductance [70,90,91]. Similarly, the proton conductance of mitochondria in intact thymocytes is reduced in UCP2 knockouts [37], and the mitochondrial membrane potential is raised in HeLa cells expressing a dominant negative UCP2 [78]. The authors conclude that UCP2 and UCP3 contribute significantly to the proton conductance of these mitochondria. However, skeletal muscle mitochondria from a human subject lacking functional UCP3 showed no alteration in coupling [92]. Subsequent studies found no decrease in the proton conductance of skeletal muscle mitochondria from UCP3 knockout mice [28,35,85] or of spleen, lung or kidney mitochondria from UCP2 knockouts [32,39]. The unchanged proton conductance in the absence of UCP2 or UCP3 is excellent evidence that these proteins do not catalyse the basal proton conductance of the mitochondria (Fig. 2a)—there is no evidence for any compensatory effect of other gene products. The earlier findings of decreased proton conductance can be explained as effects on endogenous alkenal-induced proton conductance (Figs. 2b, c, f), which we propose was partially active in the earlier studies. This proposal is supported for the cellular studies by the observation that removal of endogenous superoxide in intact thymocytes decreases the observed proton conductance and eliminates the effect of UCP2 knockout [32].

UCP2 or UCP3 knockout mice lose the ability to show inducible proton conductance in mitochondria from the appropriate tissue, providing the strongest evidence that this reaction is catalysed by UCP2 and UCP3. The GDP-sensitive activation of proton conductance by superoxide [28] or hydroxynonenal [35] is absent in skeletal muscle mitochondria from UCP3 knockout mice, and the GDP-sensitive activation of proton conductance by superoxide is absent in kidney mitochondria from UCP2 knockouts [32].

Thus loss-of-function studies show that UCP2 and UCP3 are not responsible for the basal proton conductance of mitochondria (Fig. 2a). However, they demonstrate very clearly that UCP2 and UCP3 catalyse a net proton conductance of the mitochondrial inner membrane, but only when their activity is induced by appropriate activators such as superoxide or hydroxynonenal (Figs. 2b, c, f).

3. UCPs catalyse transport of fatty acids and other anions

3.1. Anion transport by UCP2 and UCP3 in proteoliposomes and mitochondria

Measurement of the transport of fatty acid and other anions in proteoliposomes shows that UCP2 and UCP3, like UCP1 [15], can transport anions in a nucleotide-sensitive reaction (Fig. 2d) [60,63].

Rat kidney mitochondria swell in isotonic solutions of potassium chloride, nitrate or thiocyanate in the presence of valinomycin, demonstrating that they can transport monovalent anions across the membrane. GDP inhibits swelling (Echtay, K.S. and Brand, M.D., unpublished), suggesting that UCP2, like UCP1, transports anions across the mitochondrial inner membrane.

Whether fatty acid cycling explains net proton transport (Fig. 2c) is controversial for UCP1 [6,15–18,38,57]. By extension, it is also controversial for UCP2 and UCP3. Proteoliposomes containing UCP2 or UCP3 transport anions in the absence of ubiquinone but require ubiquinone for net proton transport [27], suggesting that the two processes are different. If alkylsulphonates activate the proton conductance of UCP1 in the absence of fatty acids [38], perhaps all UCPs catalyse net proton transport by some undefined mechanism that is independent of the carboxylic acid group of fatty acids.

Thus, UCP2 and UCP3 probably transport anions across the mitochondrial membrane, but the relevance of this reaction to the inducible proton conductance they catalyse has yet to be resolved.

3.2. UCP2 and UCP3 catalyse transport of fatty acid hydroperoxides across the mitochondrial inner membrane

It has been suggested that UCP2 and UCP3 export fatty acid hydroperoxide anions (Fig. 2e) [25]. In an extension of this hypothesis, if exported fatty acid hydroperoxides could protonate and flip back across the membrane (Fig. 2f), a net proton transport would be catalysed by the UCPs [93]. The two models are

distinguished by the observation that addition of synthesized linoleic acid hydroperoxides causes fast acidification of the interior of UCP2 proteoliposomes, suggesting flip of the protonated fatty acid hydroperoxide across the bilayer [93]. There is no other experimental evidence for these hypotheses.

4. Conclusions

There is no persuasive evidence that UCP2 and UCP3 catalyse the basal proton conductance of mitochondria in the absence of specific activators. On the contrary, the unchanged basal conductance in the absence of UCP2 or UCP3 in mitochondria from knockout mice provides excellent evidence that they do not. However, they do increase the proton conductance of the inner membrane when activated by products of ROS metabolism such as hydroxynonenal. The proton conductance catalysed by these proteins is strongly inhibited by purine nucleotides. How they function in the presence of physiological concentrations of ATP is yet to be elucidated; perhaps nucleotide inhibition is diminished by the presence of fatty acids, as proposed for UCP1 [13,14]. The UCPs probably also catalyse export of anions from the matrix, but whether anion export and net proton transport are related, and what the mechanism of the net proton transport reaction may be, remain unresolved. Net proton transport could involve protonation and deprotonation of the carboxylic acid group of exogenous fatty acids, hydroxide anion export, a cycle of export of fatty acid or fatty acid hydroperoxide anions followed by protonation and flip back of the acid into the matrix, or some other mechanism.

The identification of reactions catalysed by UCP2 and UCP3 is an important step towards understanding the physiological functions of these proteins in attenuation of the production of reactive oxygen species, thermogenesis and other processes, and the role of UCP2 in insulin secretion. These issues are discussed in [11].

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