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Mitochondrial H^+ Leak and Thermogenesis

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Abstract

Mitochondria of all tissues convert various metabolic substrates into two forms of energy: ATP and heat. Historically, the primary focus of research in mitochondrial bioenergetics was on the mechanisms of ATP production, while mitochondrial thermogenesis received significantly less attention. Nevertheless, mitochondrial heat production is crucial for the maintenance of body temperature, regulation of the pace of metabolism, and prevention of oxidative damage to mitochondria and the cell. In addition, mitochondrial thermogenesis has gained significance as a pharmacological target for treating metabolic disorders. Mitochondria produce heat as the result of H^+ leak across their inner membrane. This review provides a critical assessment of the current field of mitochondrial H^+ leak and thermogenesis, with a focus on the molecular mechanisms involved in the function and regulation of uncoupling protein 1 and the ADP/ATP carrier, the two proteins that mediate mitochondrial H^+ leak.

ATP: adenosine 5′-triphosphate

H^+ leak: passive H^+ conductance of the inner mitochondrial membrane that uncouples H^+ transport activities of the ETC and ATP synthase

IMM: inner mitochondrial membrane

ETC: electron transport chain

Brown fat: specialized thermogenic tissue responsible for adaptive thermogenesis

INTRODUCTION

Mitochondria are intracellular organelles, bound by two membranes, that supply the eukaryotic cell with energy. Together with the upstream energy metabolism pathways, mitochondria convert diverse sources of chemical energy (nutrients) into adenosine 5′ triphosphate (ATP) and heat. Despite being often overlooked and understudied as compared to mitochondrial ATP production, mitochondrial heat production (thermogenesis) has crucial physiological functions. It supports core body temperature, regulates body weight by setting a balance between energy consumption and expenditure, and controls mitochondrial production of free radicals to preserve mitochondrial and cell integrity. Mitochondrial thermogenesis is sometimes represented as a by-product of mitochondrial ATP production, resulting from the inherent inefficiency of mitochondrial ATP production pathways. Although the mitochondrial ATP production machinery is not 100% efficient and thus dissipates some heat, this inefficiency is not the topic of this review. Here, we consider a specialized thermogenic pathway associated with a regulated H^+ leak across the inner mitochondrial membrane (IMM), which draws energy from the mitochondrial electrochemical H^+ gradient in parallel to the ATP production pathway.

The subject of mitochondrial H^+ leak and thermogenesis holds significant promise in terms of addressing metabolic disorders and mitochondrial dysfunction. Unfortunately, the same field has also been an area of intense controversy. Traditionally, mitochondrial H^+ leak was addressed with two very different approaches. One approach was based on measurements of mitochondrial uncoupled respiration (uncoupled means not associated with ATP production). This method studied mitochondrial H^+ leak in intact mitochondria or cells but was indirect [i.e., not measuring H^+ leak but rather an associated oxygen consumption by the electron transport chain (ETC)] and offered very limited control over the experimental conditions. The second method employed reconstitution of the proteins mediating mitochondrial H^+ leak in artificial lipid bilayers to measure their activity more directly and under more controlled conditions; however, these assays were criticized for lacking the native membrane environment. These two assays helped establish the phenomenon of mitochondrial H^+ leak and thermogenesis, but their limitations resulted in controversies and often misunderstanding of mitochondrial H^+ leak. Therefore, we introduced a different method to study mitochondrial H^+ leak: its direct patch-clamp measurement across the whole IMM. This method combines the best qualities of the two classical methods while avoiding most of their pitfalls. It studies the proteins mediating mitochondrial H^+ leak in the native membrane environment, directly and under perfectly controlled experimental conditions. The patch-clamp technique is universally considered a method of choice for understanding membrane transport proteins.

This review provides a critical assessment of the study of mitochondrial H^+ leak and thermogenesis in light of recent findings enabled by the mitochondrial patch-clamp. This review is not intended to be comprehensive but aims to address and reevaluate some of the most essential aspects and implications of mitochondrial H^+ leak.

MITOCHONDRIA AS ORGANELLES THAT GENERATE ATP AND HEAT

Mitochondria: Beyond ATP Production

Mitochondria are intracellular organelles primarily known for producing ATP, the universal energy source of the cell. However, all mitochondria also produce heat. Arguably, it would even be difficult to claim that ATP is the dominant form of energy produced by mitochondria. This is because mitochondria of the specialized thermogenic tissue brown fat function specifically to produce heat (1, 2). However, even mitochondria of tissues other than brown fat, such as skeletal muscle, heart, and liver, can convert as much as 20% of nutrient energy into heat (3).

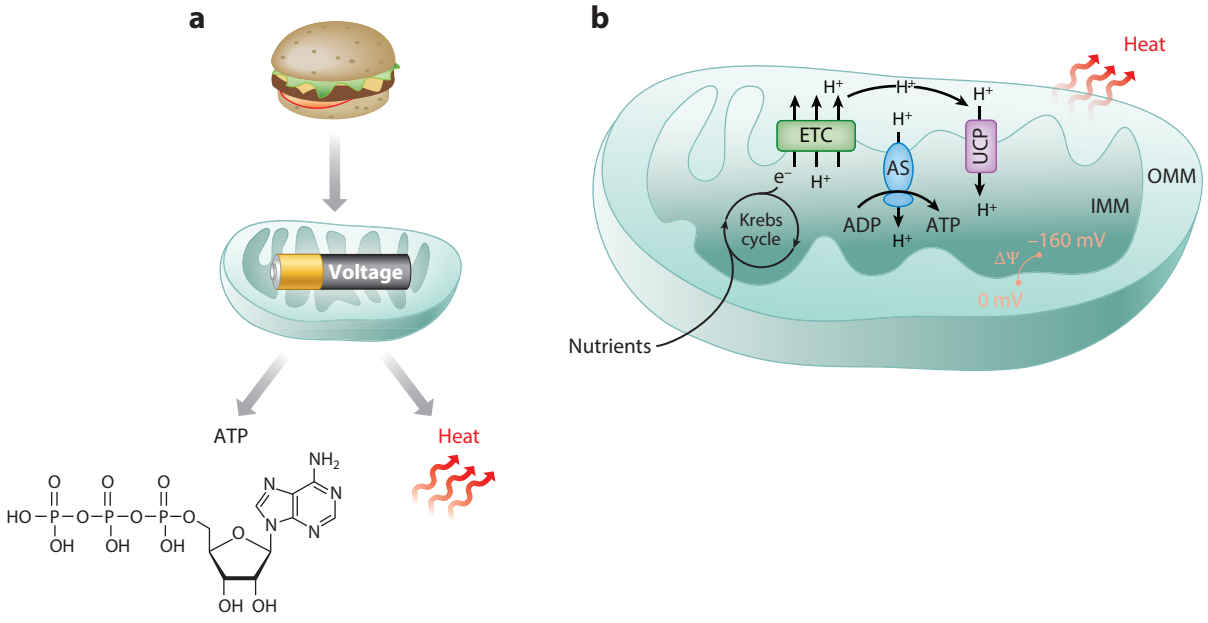

Figure 1

Mitochondrial adenosine 5′ triphosphate (ATP) and heat production. (*a*) General diagram of energy conversion within mitochondria. The chemical energy of nutrients is converted into a mitochondrial voltage, which is then converted into ATP and heat. (*b*) Mechanism of mitochondrial ATP and heat production. Mitochondria have two membranes, the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM). The OMM is freely permeable for ions and small metabolites with a molecular weight <2,000 Da but presents a barrier for the transport of proteins. In contrast, the IMM permeability is tightly controlled for all ions, metabolites, and proteins. The IMM contains the machinery for ATP synthesis and confines the enzymes of the Krebs cycle within the mitochondrial matrix. The electron transport chain (ETC) uses the energy of electrons supplied by the Krebs cycle (in the form of electron donors NADH and $FADH_2$) to pump H^+ outside the IMM and generates membrane potential ($\Delta\Psi$) across the IMM. $\Delta\Psi$ is then used by the ATP synthase (AS) to generate ATP and by uncoupling proteins (UCPs) to generate heat.

A simplified scheme of mitochondrial energy conversion is shown in **Figure 1**. Mitochondria convert the chemical energy of nutrients into electrical voltage, which is then converted into ATP and heat. At a more mechanistic level, in the first step in mitochondrial energy conversion, nutrients enter the Krebs cycle and are oxidized to generate high-energy electron donors NADH and $FADH_2$. In the second step, NADH and $FADH_2$ donate their high-energy electrons to the mitochondrial ETC that eventually passes them onto oxygen where they have significantly lower energy. The ETC uses the energy released in such electron transfer to pump H^+ outside the mitochondrial matrix and to generate an electrochemical H^+ gradient across the IMM. This gradient primarily consists of voltage across the IMM [membrane potential ($\Delta\Psi$), approximately −160 mV in the mitochondrial matrix as compared to cytosol], while its chemical component is relatively small (ΔpH ~0.5) (4). In the last step, $\Delta\Psi$ is converted into ATP and heat. ATP is generated by ATP synthase that passes H^+ down $\Delta\Psi$ and uses the released energy to generate ATP from adenosine 5′-diphosphate (ADP) and inorganic phosphate. In contrast, heat is generated by so-called uncoupling proteins (UCPs) that also pass H^+ down $\Delta\Psi$ but do not generate ATP, instead letting energy dissipate as heat (**Figure 1**).

UCPs are called uncoupling proteins because they break a perfect coupling between the H^+ flows via the ETC and ATP synthase. The uncoupling H^+ current across the IMM mediated by UCPs is called H^+ leak. Free fatty acids (FAs) are the physiological activators of H^+ leak via UCPs (5).

ADP: adenosine 5′-diphosphate

UCP: uncoupling protein of the IMM responsible for H^+ leak and thermogenesis; this term does not necessarily mean UCP1–UCP4

Uncoupling: dissociation between H^+ transport activities of the ETC and ATP synthase due to passive conductance of the IMM for certain ions

FA: free fatty acid

UCP1: uncoupling protein 1; responsible for H^+ leak and thermogenesis in brown and beige fat

DNP: 2,4-dinitrophenol; protonophore and mitochondrial uncoupler

FCCP: cyanide-4-(trifluoromethoxy) phenylhydrazone; protonophore and mitochondrial uncoupler

PTP: permeability transition pore; a large-conductance nonselective pore activated in the IMM under certain conditions

The mechanisms of mitochondrial H^+ leak are poorly understood. Although uncoupling protein 1 (UCP1) has been established as the UCP responsible for mitochondrial H^+ leak in the specialized thermogenic tissues brown fat and beige fat (1, 6–10), the molecular identity of the UCP(s) of all other tissues is actively debated. Even for UCP1, the mechanism by which FAs activate H^+ leak is elusive, and several conflicting models have been proposed (1).

Because the molecular mechanisms of the native mitochondrial H^+ leak remained largely unknown and its pharmacological control impossible, H^+ conductance of the IMM was induced artificially using chemical protonophores such 2,4-dinitrophenol (DNP) and cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP). This helped to reveal the physiological significance of the mitochondrial H^+ leak. FCCP and DNP are hydrophobic, membrane-soluble weak acids that can induce H^+ leak by carrying H^+ across the IMM without the help of membrane transport proteins (11, 12). Although FCCP was primarily used in laboratory research, DNP initially found broad application in the clinic. DNP was used as an explosive during World War I, when its thermogenic effect was serendipitously discovered at munition factories (13). DNP was then widely used in humans in the 1930s and 1960s and became known as one of the most efficient weight loss medications. Indeed, DNP increases the metabolic rate, shifting the energy balance toward expenditure and reducing fat deposits (13). Although no significant safety concerns were identified originally (14), side effects, especially cataracts and even overdose-associated deaths, were reported later, and the drug was then banned by the US Food and Drug Administration (15). Recently, there has been a significant resurgence of interest in DNP with the discovery that it is extraordinarily efficient against type 2 diabetes, fatty liver disease, and metabolic syndrome in animal models (16). However, the use of DNP in humans remains prohibited.

Controlling the mitochondrial H^+ leak for therapeutic purposes holds significant promise, but its realization depends on the identification of UCPs and understanding the molecular mechanism of their operation.

Mitochondrial H^+ Leak Versus Mitochondrial Uncoupling

Although the transport proteins of the IMM responsible for H^+ leak are called uncoupling proteins, it should be mentioned that mitochondrial uncoupling can be caused not only by H^+ leak. Increasing conductance of the IMM for any type of ion will dissipate $\Delta\Psi$ and uncouple the activities of the ETC and ATP synthase. Among notable examples, the IMM channel known as mitochondrial Ca^{2+} uniporter (MCU) can dissipate $\Delta\Psi$ and induce mitochondrial uncoupling (4). Other ion channels have been reported in the IMM, including several types of K^+ channels and the permeability transition pore (PTP) (17, 18). Any of them, even if they have a low open-state probability, can allow abundant cytosolic K^+ to enter mitochondria and uncouple them. Finally, certain electrogenic transporters of the IMM may also dissipate $\Delta\Psi$. All these modes of electrogenic transport will reduce the efficiency of mitochondrial ATP synthesis and make mitochondria generate more heat.

However, among all electrogenic conductances of the IMM involved in mitochondrial uncoupling, only H^+ leak is a specialized thermogenic mechanism. The rest have other primary physiological functions, such as Ca^{2+}-dependent stimulation of the Krebs cycle (MCU) or regulation of mitochondrial cell death (PTP), with uncoupling and the associated thermogenesis serving as by-products of these primary functions. The central role of H^+ leak in thermogenesis is illustrated by the specialized thermogenic tissue brown fat where mitochondrial heat production is due to H^+ leak via UCP1. H^+ leak is the simplest way to connect the H^+ transport activity of the ETC with thermogenesis, resulting in a futile cycling of H^+ across the IMM that causes mitochondria to generate heat.

We mention the alternative mitochondrial uncoupling pathways in this review because they introduce significant complexity into mitochondrial bioenergetics and could be confused with H^+ leak when indirect methods are used.

Methods to Study Mitochondrial H^+ Leak

There have been two main approaches to studying mitochondrial H^+ leak. The first measures the rate of mitochondrial uncoupled respiration in either isolated mitochondria or intact cells. In this assay, oligomycin, a highly specific inhibitor of ATP synthase, is used to eliminate H^+ flow through the ATP synthase. Under these conditions, the outward H^+ flux generated by the ETC should return back to the matrix only via H^+ leak, and thus, the oxygen consumption by the ETC reflects the magnitude of H^+ leak (2, 19) (**Figure 1*b***). The benefit of this method is that it studies H^+ leak in the intact native system (mitochondria or cells). However, H^+ leak is not measured directly, and a change in the respiration rate of the ETC, an enormous molecular complex, can be caused by other factors. The assumption that the uncoupled respiration of the ETC is always associated with H^+ leak is questionable, as other IMM conductances also are capable of uncoupling, such as K^+, PTP, or even compromised IMM integrity. Finally, the experimental conditions such as the compositions of matrix and cytosolic solutions, concentration of membrane FAs, pH gradient across the IMM, and $\Delta\Psi$ are poorly controlled in respiration studies, and their variation during the experiment can further complicate the interpretation of the results.

Another classical approach attempts to recapitulate the phenomenon of mitochondrial H^+ leak in artificial membranes. In this approach, candidate UCPs are reconstituted in lipid bilayers or liposomes and the properties of the resultant H^+ leak are studied (20, 21). This method helped to reduce the complexity of the system, achieve more direct measurements of H^+ leak, and improve control over the experimental conditions. However, this method was criticized because the UCPs are not studied in their native membrane environment, potentially altering their functional properties and possibly even assigning the uncoupling role to proteins that do not mediate H^+ leak in vivo. Although the mitochondrial respiration studies and the reconstitution studies laid important foundations for our understanding of mitochondrial H^+ leak, the data collected with these two methods often supported very different conclusions, resulting in controversies.

Another method for studying mitochondrial H^+ leak has recently emerged. It was demonstrated that patch-clamp electrophysiology can be used to directly measure H^+ leak across the whole IMM of isolated mitochondria devoid of the outer membrane (mitoplasts) (8, 22–24). The patch-clamp technique is the gold standard in studies of membrane transport proteins. The vast experience of applying this method in neuroscience and cardiac and muscle physiology demonstrates that it generates reproducible and physiologically relevant results. In mitochondria, it measures H^+ leak in the native IMM, with exceptional amplitude and time resolution as well as reliable control of all critical experimental conditions such as transmembrane voltage and solution compositions on both sides of the IMM. This method can record mitochondrial H^+ leak in isolation from other IMM conductances, thus distinguishing it from K^+ channels, PTP, or compromised membrane integrity.

Therefore, the patch-clamp electrophysiology approach has eliminated all major disadvantages associated with the respiration and reconstitution approaches, but it must be considered that the IMM cristae are likely to unfold during mitoplast preparation, making the IMM less intact compared to its state in the respiration studies. In this review, we often compare the results obtained with these three methods to provide better insight into the molecular mechanisms of mitochondrial H^+ leak and address the decades-long controversies in this field.

GDP: guanosine 5′-diphosphate

AAC: ADP/ATP carrier, also referred to as adenine nucleotide translocator (ANT)

SLC25: the superfamily of mitochondrial solute carriers

MITOCHONDRIAL UNCOUPLING PROTEINS

Historical Perspective on Mitochondrial Uncoupling Proteins

UCP1 was the first UCP identified (25, 26). It has been unambiguously demonstrated to mediate mitochondrial H^+ leak in the specialized thermogenic tissues brown fat (6, 7, 9, 25, 27) and beige fat (8, 10) by studies using the UCP1 inhibitor guanosine 5′-diphosphate (GDP) and a UCP1-deficient mouse model. However, because UCP1 expression is limited to brown and beige fat, the UCPs of other tissues remained unknown.

Soon after the identification of UCP1, it was discovered the FA-induced uncoupled respiration in isolated skeletal muscle and liver mitochondria is reduced by carboxyatractyloside (CATR), a highly specific inhibitor of the mitochondrial ADP/ATP carrier (AAC) (28–30). These results indicated that AAC, which is responsible for the exchange of matrix ATP for cytosolic ADP (31), may also mediate H^+ leak in the presence of FAs, the physiological activators of mitochondrial H^+ leak and thermogenesis. However, around the same time, AAC was also implicated as the PTP (32–34), a large-conductance nonselective pore activated under certain conditions in the IMM. Similar to H^+ leak, the PTP is induced by FAs and uncouples mitochondria (35, 36). Thus, it was difficult to unambiguously determine whether AAC induces mitochondrial uncoupling via a selective H^+ leak, nonselective PTP, or both.

Genetic evidence for the involvement of AAC in mitochondrial H^+ leak used to be scarce. Humans have four AAC isoforms, encoded by four different genes, *AAC1–AAC4* (31, 37, 38). The *AAC3* gene is absent in mice (39, 40). The existence of several AAC isoforms with likely redundant functions has complicated analysis of knockout phenotypes. In addition, the primary focus was on the roles of AAC in ADP/ATP exchange and the PTP. As a result, the loss of ADP/ATP exchange and disruption of mitochondrial ATP production was confirmed along with an important but perhaps nonessential role of AAC in the PTP (40–43). However, the involvement of AAC in mitochondrial H^+ leak was only partially addressed. In an *AAC1* knockout mouse, only basal mitochondrial uncoupled respiration (without FA added) was measured, and contrasting results were reported, from no effect to a decrease (40–42). Double *AAC1/AAC2* knockout in mouse liver unexpectedly led to increased basal mitochondrial uncoupled respiration (43). Such a discrepancy is not surprising given the fundamental role of AAC in mitochondrial physiology. The broad effects of AAC knockouts on mitochondrial function (42) can give rise to multiple phenotypes depending on the experimental conditions used. Thus, the involvement of AAC in mitochondrial H^+ leak, especially FA-induced H^+ leak, remained unclear.

The implication of UCP1 and AAC in H^+ leak was followed by suggestions that other mitochondrial transporters, such as the phosphate carrier (44, 45), aspartate-glutamate carrier (46), and dicarboxylate carrier (46–48), could also facilitate FA-induced mitochondrial H^+ leak. All of these transporters belong to the solute carrier 25 (SLC25) family of mitochondrial anion carriers and are related to AAC and UCP1. However, the pharmacology used to demonstrate the involvement of these three additional carriers in H^+ leak was not specific. In addition, similar to AAC, the phosphate carrier was also implicated in the PTP (49), complicating its functional analysis. Mouse models deficient for phosphate and aspartate-glutamate carriers have been generated, but similar to the AAC knockouts, the focus was not on H^+ leak but other transport modes of these proteins (50–55). Some of these studies measured basal uncoupled respiration, but no significant abnormalities in carrier-deficient models were observed to implicate the phosphate or aspartate-glutamate carrier in H^+ leak or mitochondrial uncoupling (51, 54, 55). In addition to its role in transporting inorganic phosphate, knockout of the phosphate carrier revealed its possible involvement in the PTP (50). To the best of our knowledge, no dicarboxylate carrier knockout mice have been reported.

With the onset of the genomic era, mitochondrial carriers UCP2 (56, 57) and UCP3 (58–60) were identified as close UCP1 homologs. In contrast to UCP1, UCP2 and UCP3 are expressed ubiquitously and thus could mediate mitochondrial uncoupling in tissues other than brown fat. By manipulating the levels of UCP2/UCP3 expression (genetic ablation or overexpression) or using the UCP inhibitor GDP, numerous studies implicated UCP2 and UCP3 in mitochondrial H^+ leak and uncoupling. UCP2 and UCP3 were also shown to facilitate H^+ leak after reconstitution in artificial phospholipid membranes (61, 62). However, several other studies did not support that UCP2 and UCP3 are bona fide UCPs (reviewed in 63–66). Despite the controversy, UCP2 and UCP3 are still generally believed to mediate mitochondrial H^+ leak, at least under certain conditions, and the literature elaborating on their involvement in mitochondrial uncoupling is immense. Apart from their putative roles in mediating mitochondrial H^+ leak, UCP2 and UCP3 have also been implicated in metabolite transport and regulation of glucose and glutamine oxidation (67–69).

UCP2: uncoupling protein 2; a protein of the SLC25 family and a close homolog of UCP1

UCP3: uncoupling protein 3; a protein of the SLC25 family and a close homolog of UCP1

Because the UCP candidates discussed above are all members of the SLC25 superfamily of mitochondrial solute carriers, it has even been suggested that all ~50 members of this family can contribute to H^+ leak (3, 70).

To summarize, while UCP1 was confirmed to be the bona fide mitochondrial UCP responsible for mitochondrial H^+ leak and thermogenesis in brown/beige fat, the identity and number of UCPs in other tissues remained elusive. An important barrier to the identification of the UCP(s) in tissues other than brown fat was that all candidate proteins had other major transport functions in addition to the putative H^+-transport activity. Thus, their knockout could have very broad effects on mitochondrial function, including mitochondrial respiration; thus, the respiration assays were not well suited to study H^+ leak specifically. The situation was further complicated by the fact that H^+ leak in tissues other than brown fat is not robust enough to be easily detected on the background of other possible changes. Given the complexity of the mitochondrial transport of ions and metabolites, addressing the molecular mechanisms of the mitochondrial H^+ leak required the development of a method for its direct measurement.

UCP1: Mitochondrial Uncoupling Protein of Specialized Thermogenic Tissues

Brown adipose tissue is the main location for nonshivering adaptive thermogenesis in response to stimuli such as cold or diet, and it is essential for body temperature maintenance (1, 71). Research to understand the mechanisms of nonshivering adaptive thermogenesis has been energized by the enormous interest in human metabolic diseases linked to obesity, including type 2 diabetes and fatty liver disease (72). Indeed, brown fat possesses unique mitochondria that are extremely efficient in heat production and use fat as a preferred source of energy. These mitochondria express the uncoupling protein UCP1, a sophisticated H^+ leak pathway responsible for brown fat thermogenesis.

The unusual behavior of isolated brown fat mitochondria was first detected in the 1960s (73). In contrast to mitochondria of other tissues, brown fat mitochondria were profoundly uncoupled when isolated using standard procedures (73). It was then determined that these mitochondria can be recoupled by adding purine nucleotides such as GDP or by removing free FAs with albumin (74). Eventually, FAs and purine nucleotides were determined to be the principal physiological regulators of a putative protein (referred to as thermogenin or UCP) responsible for H^+ leak across the IMM of brown fat (1, 74, 75). This set the stage for attempts to identify this protein, which eventually was accomplished by the Klingenberg and Ricquier groups (6, 9). Brown fat UCP was a protein with six predicted transmembrane helixes, which could be grouped into three homologous repeats of two helixes each. Surprisingly, it was homologous to the previously identified

Beige adipocytes: thermogenic brown-fat-like adipocytes in white fat; despite the morphological and functional similarities with brown fat, they have a different cellular origin

AAC (6), establishing the SLC25 family of mitochondrial solute carriers. The protein was later renamed UCP1 upon the identification of UCP2 and UCP3, two other SLC25 members. The molecular identification of UCP1 helped to clarify the mechanistic basis of its purine nucleotide inhibition (76), but the mechanism by which UCP1 facilitates H^+ transport in the presence of free long-chain FAs has been one of the most controversial topics in the field of bioenergetics (70, 76, 77). In fact, the actual transport function of UCP1 is still debated, as in some models UCP1 transports FA anions rather than H^+ (70). The mechanism by which UCP1 facilitates H^+ leak in the presence of FAs is addressed in subsequent sections of this review.

Direct patch-clamp recording from the IMM of brown fat demonstrated that the UCP1-mediated H^+ current is quite large (23). In fact, it is one of the largest H^+ conductances measured across any biological membrane, and in this respect, it can be compared only to the currents mediated by voltage-gated H^+ channel H_v1 (78). However, UCP1 is a transporter that should have a significantly slower H^+ turnover rate as compared to an H^+ channel. The large H^+ current mediated by UCP1 is due to the extremely high density of this protein in the IMM of brown fat (25). The FA-dependent H^+ current across the IMM of brown fat is eliminated in UCP1 knockout mice (23).

Originally, brown fat was considered the only site for adaptive thermogenesis. However, it was later discovered that under certain conditions, such as prolonged cold exposure, increased sympathetic tone, or physical exercise, white fat develops islands of cells with the morphology of brown adipocytes. Instead of a single large lipid droplet and scarce mitochondria (monolocular adipocytes, characteristic of white fat), these cells contain numerous small lipid droplets and abundant mitochondria that give them a brownish appearance (so-called multilocular adipocytes, characteristic of brown fat). Thus, these multilocular cells were named beige (or brite) adipocytes (79–82). Early on, it was suggested that all beige adipocytes express UCP1, and UCP1 was universally used as a marker to distinguish beige adipocytes from white adipocytes within white fat depots. Also, it was generally accepted that all beige adipocytes were capable of UCP1-dependent mitochondrial thermogenesis. However, direct patch-clamp characterization of H^+ leak across the IMM of subcutaneous and abdominal beige fat demonstrated the existence of both UCP1-positive and UCP1-negative beige adipocytes (8). All mitochondria from subcutaneous (inguinal) beige fat, when analyzed with patch-clamp electrophysiology, had UCP1-dependent H^+ leak similar to that found in brown fat. In contrast, only about 15% of the mitochondria in abdominal (epididymal) beige fat had the UCP1-dependent H^+ leak, with UCP1 currents being undetectable in the remaining 85% (8). Thus, mitochondrial patch-clamp analysis revealed a new population of beige adipocytes that do not use UCP1 for thermogenesis (8). In mice, the UCP1-negative beige adipocytes are abundant in abdominal fat, whereas UCP1-positive beige adipocytes are the predominant type in subcutaneous fat. The UCP1-negative and -positive beige adipocytes share significant similarities such as being multilocular and having a large mitochondrial network, a robust thermogenic gene program, and a distinctive oxidative phosphorylation profile different from that of brown fat (8). Moreover, both UCP1-negative and -positive beige adipocytes are capable of heat production via a futile cycle of creatine phosphorylation/dephosphorylation (8, 71, 83).

Thus, UCP1 was unambiguously identified as a UCP of specialized thermogenic tissues, brown and beige fat. However, UCP1-dependent thermogenesis is not necessarily the only thermogenic mechanism present in beige fat. Indeed, not all beige adipocytes express UCP1, and beige fat possesses a futile cycle of creatine phosphorylation/dephosphorylation as an alternative thermogenic mechanism. However, UCP1 is by far the dominant mechanism of adaptive thermogenesis in beige and brown fat, and its deficiency leads to profound disruption of thermoregulation and diet-induced thermogenesis in mice.

AAC: Ubiquitous Mitochondrial Uncoupling Protein

H^+ leak is not a distinctive property of the mitochondria of the specialized thermogenic tissues. Even though thermogenesis is not the primary function of other somatic tissues such as skeletal muscle and liver, their mitochondria still have H^+ leak. It has been estimated that H^+ leak could be responsible for up to 52% of the total mitochondrial oxygen consumption in resting rat skeletal muscle (84, 85) and for up to 26% in resting (not engaged in gluconeogenesis and ureagenesis) rat hepatocytes (86). These numbers are reduced to 34% and 22%, respectively, as the activity of these tissues increases (85), but this still demonstrates that mitochondrial H^+ leak and thermogenesis can have a profound effect on the bioenergetics of these tissues and of organisms in general. It was estimated that the mitochondrial H^+ leak of all tissues combined could represent as much as 20% of the resting metabolic rate of the entire body (85).

Although H^+ leak is an essential aspect of mitochondrial physiology, the molecular identity of transport protein(s) mediating the thermogenic H^+ leak across the IMM in tissues other than brown/beige fat remained elusive for decades. Recently, the patch-clamp technique was applied to directly measure H^+ leak across the whole IMM of tissues other than brown fat, including skeletal muscle, heart, liver, and kidney. The results showed that the mitochondria of all these tissues possess a robust FA-induced H^+ leak (22), supporting the previous data demonstrating that mitochondria of all tissues are capable of thermogenesis and that heat is an essential component of mitochondrial energy output.

Most importantly, the patch-clamp technique helped determine the molecular identity of the mitochondrial UCP in nonadipose tissues. Being the closest UCP1 homologs, the ubiquitously expressed UCP2 and UCP3 have received much attention (64), but their contributions to mitochondrial uncoupling have remained controversial (63, 67). Electrophysiological analysis demonstrated that the amplitude of the FA-induced H^+ leak in skeletal muscle mitoplasts isolated from wild-type, *UCP2*$^{-/-}$ and *UCP3*$^{-/-}$ mice were comparable. Additionally, the UCP inhibitor GDP (61, 63, 64) had no effect on the H^+ leak. Thus, UCP2 and UCP3 do not contribute significantly to H^+ leak (22).

Strikingly, the electrophysiological analysis demonstrated that AAC is the protein responsible for the FA-induced mitochondrial H^+ leak in skeletal muscle, heart, liver, and kidney. Indeed, an AAC-specific inhibitor CATR inhibited FA-induced H^+ leak in all of these tissues. Moreover, another specific AAC inhibitor, bongkrekic acid, abolished H^+ leak in skeletal muscle and heart (22). This pharmacological identification was reinforced by the measurement of H^+ leak in *AAC1*- and *AAC2*-deficient mice. AAC1 and AAC2 are the two somatic isoforms of AAC in mice. The third somatic isoform, AAC3, is present in humans but absent in mice (39, 40), whereas AAC4 is expressed mainly in germ cells and pluripotent stem cells (38). AAC1 expression is highest in heart and skeletal muscle, and AAC2 is most abundant in kidney (39, 87). In *AAC1*$^{-/-}$ mice (40), FA-induced H^+ leak completely disappeared in heart mitoplasts and was reduced about 70% in skeletal muscle (22). Importantly, the remaining H^+ leak in skeletal muscle was CATR-sensitive, suggesting the involvement of AAC2 in H^+ leak. AAC1 knockout did not result in reduction of H^+ leak in kidney, a tissue with significant expression of AAC2, while H^+ leak was still inhibited by CATR (22). In AAC2 hypomorphic mice (87), in which AAC2 was considerably reduced but not completely eliminated, FA-induced H^+ leak was not significantly altered in heart and kidney (22), likely due to compensation by AAC1, as previously observed in these mice (87). These results indicate that AAC1 plays a crucial role in H^+ leak, at least in heart and skeletal muscle. Although the reduction of H^+ leak in AAC2 hypomorphic mice was not significant, the recording of robust CATR-sensitive H^+ leak in skeletal muscle of *AAC1*$^{-/-}$ mice demonstrates that AAC2 is capable of mediating H^+ leak. Thus, direct patch-clamp recording from the IMM demonstrated that AAC is responsible for the FA-induced mitochondrial H^+ leak.

Respiration experiments in isolated mitochondria and intact cells further confirmed the crucial role of AAC in mitochondrial H^+ leak. Uncoupled respiration induced by long-chain FAs was eliminated in cardiac mitochondria isolated from *AAC1*$^{-/-}$ mice (22). The role of AAC in mitochondrial H^+ leak and uncoupled respiration was also analyzed in a C2C12 mouse cell line deficient for AAC1 and AAC2 (AAC1/AAC2 double knockout). Despite being fully AAC deficient, double knockout cells had unaltered mitochondrial biomass and their mitochondria had robust respiratory capacity. The basal and FA-dependent uncoupled respiration rates were dramatically reduced in both isolated double knockout mitochondria and intact cells (22). The dramatic effect of AAC deficiency on mitochondrial uncoupled respiration confirms the conclusion from the electrophysiological experiments that AAC is responsible for mitochondrial H^+ leak in tissues other than brown/beige fat. Thus, there are two bona fide thermogenic UCPs: ubiquitous AAC and fat-specific UCP1.

Previously, AAC was proposed as a candidate mitochondrial UCP in tissues other than brown/beige fat, but assigning this functional identity to AAC unambiguously was difficult based on the indirect H^+ leak measurements using mitochondrial respiration. AAC was first implicated in mitochondrial uncoupling by Skulachev's group (29) based on the observation that CATR inhibits FA-induced mitochondrial uncoupled respiration in skeletal muscle and liver. Later, the Brand group (41) proposed that basal uncoupled respiration (a small uncoupled respiration observed in the presence of FA acceptor albumin that is assumed to be FA-independent) is also caused by AAC. However, these experiments fell short at convincing the field that AAC operates as a bona fide UCP. First, the inhibition of mitochondrial uncoupling by CATR was often partial (88, 89). Second, in addition to H^+ leak, AAC was implicated in the PTP (4), which is also activated by FAs and causes mitochondrial uncoupling. Furthermore, because AAC is one of the most abundant proteins in the IMM, changes in the AAC conformational state induced by AAC ligands (e.g., CATR) can vastly affect mitochondrial morphology (condensed versus orthodox) and potentially change respiration rates (31, 90). Finally, the degree of AAC contribution to the H^+ leak as compared to other possible UCPs remained uncertain. Mitochondrial proteins such as the phosphate carrier (44, 45), aspartate-glutamate carrier (46, 91), dicarboxylate carrier (46–48), UCP2 and UCP3 (64, 66), and eventually all members of the SLC25 family were implicated in mitochondrial H^+ leak (3). The uncertainties in the interpretation of respiration data and the numerous possible UCP candidates have confused the field and distracted attention from AAC.

The direct patch-clamp measurements of mitochondrial H^+ leak across the native IMM were instrumental in reevaluating the identity of the protein(s) that control mitochondrial uncoupling in tissues other than brown and beige fat. In the patch-clamp measurements, the so-called basal H^+ leak was not observed in the absence of long-chain FAs, while application of FA induced a robust H^+ current across the IMM that appeared to be solely mediated by AAC based on pharmacological and knockout experiments (22). Interestingly, the patch-clamp experiments demonstrated that although long-chain FAs can activate H^+ leak via AAC in both the "c" and "m" conformational states, CATR only inhibits H^+ leak in the c-state (CATR is a c-state specific inhibitor) (22). This could explain, at least partially, why CATR often showed incomplete inhibition of the FA-induced H^+ leak in mitochondrial respiration studies (88, 89).

The fact that AAC is the most highly expressed of all SLC25 family members could explain why this transporter is the main UCP. However, it is also obvious that if the remaining ~50 members of the SLC25 family also can mediate H^+ leak, there would be a significant AAC-independent H^+ current mediated by the combination of the remaining SLC25 members. Moreover, some SLC25 family members (such as phosphate carrier or aspartate-glutamate carrier) are expressed at levels comparable to that of AAC (92, 93). However, as recorded by patch-clamp electrophysiology, AAC dominates the FA-induced H^+ leak in skeletal muscle, heart, liver, and kidney (22).

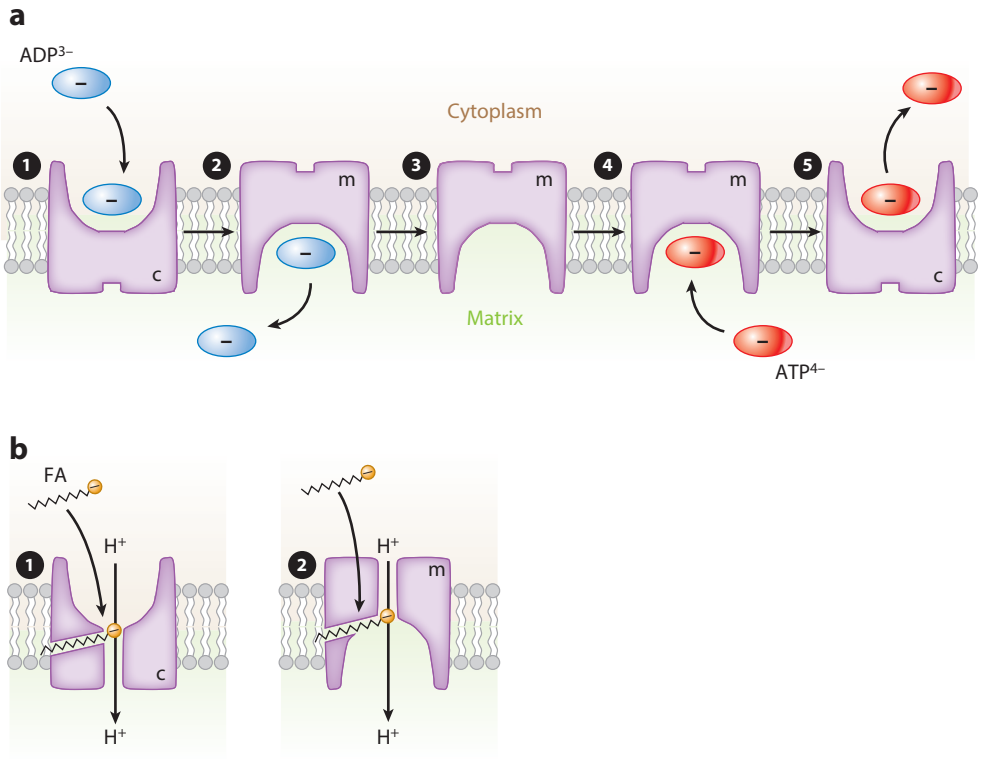

Figure 2

The two transport functions of AAC. (*a*) ADP/ATP exchange. Cytosolic Mg^{2+}-free ADP binds to the c-state of AAC (the substrate binding site exposed to the cytosol) (①), AAC changes its conformation to the m-state (the substrate binding site exposed to the matrix), and ADP dissociates into the matrix (②). AAC dwells in the m-state (③). When matrix Mg^{2+}-free ATP binds to the m-state (④), AAC changes its conformation to the c-state, and ATP dissociates into cytosol (⑤) (31). (*b*) The FA-dependent H^+ leak. When ADP and ATP are not associated with the AAC substrate binding site, cytosolic free long-chain FA can bind to either the c-state (①) or m-state (②) of AAC and activate H^+ leak. Matrix long-chain FAs cannot activate H^+ leak via AAC in either of the two states (22). Abbreviations: AAC, ADP/ATP carrier; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; FA, fatty acid.

Thus, AAC has two modes of transport, the classical ADP/ATP exchange and the FA-dependent H^+ leak (**Figure 2**). These two transport functions control the most basic aspects of mitochondrial bioenergetics, ATP production, and thermogenesis.

PHYSIOLOGICAL REGULATION OF MITOCHONDRIAL UNCOUPLING PROTEINS AND H^+ LEAK

Does Basal Mitochondrial H^+ Leak Exist?

Although long-chain FAs increase mitochondrial H^+ leak in all tissues (5, 89, 94), mitochondria remain partially uncoupled even in the presence of the FA acceptor albumin when free FAs are believed to be completely eliminated. Thus, the existence of a constitutive mitochondrial H^+ leak requiring no activators was postulated. Brand and coauthors called it basal H^+ leak and suggested that it is largely mediated by AAC and UCP1 (3, 19, 41, 95). Although the precise mechanism of

the basal H^+ leak has not been explained, it was tentatively proposed to occur via the interface between AAC/UCP1 and the lipid bilayer and to depend only on the membrane density of these proteins and not their specific transport activities (41, 95). The long-chain FAs would increase H^+ leak via UCP1 and AAC further, but through a mechanism that could be different from that of the basal H^+ leak. The existence of the basal H^+ leak would imply that the IMM is constitutively leaky for H^+, and there is a significant constitutive inefficiency in mitochondrial ATP production.

It is important to understand that the basal H^+ leak has never been measured directly but rather assumed from mitochondrial uncoupled respiration in the presence of oligomycin and the FA acceptor albumin. There is an undeniable phenomenon of mitochondrial uncoupled respiration under these conditions (so-called basal mitochondrial respiration). However, explaining such respiration through basal H^+ leak may not be accurate. Respiration studies alone are not capable of such definitive mechanistic insight, and there might be explanations for the basal mitochondrial respiration other than basal H^+ leak.

First, complete FA extraction from a suspension of isolated mitochondria using albumin would be impossible. Thus, the basal H^+ leak could simply be explained by residual FA-dependent H^+ leak. The belief that albumin can completely extract long-chain FAs from the IMM to achieve the strict absence of long-chain FAs associated with UCP1 or AAC could be overstated. Indeed, addition of a finite amount of albumin to a large number of long-chain FAs contained within a mitochondrial suspension (as done in the respiration experiments) will never achieve a zero concentration of FA. The situation is further complicated by the fact that long-chain FAs and other extractable lipids could be continuously generated within the mitochondrial membranes, and all of these lipids will eventually saturate the albumin buffer (albumin is not specific for FAs and can bind other lipids). A phospholipase activity associated with mitochondria [such as that of phospholipase A2 (PLA2)], which has been reported by several groups (8, 23, 96–98), can continuously generate free long-chain FAs from membrane phospholipids. Thus, complete extraction of long-chain FAs by albumin in respiration studies is not a certainty.

Second, the basal respiration can also be due to IMM conductances other than H^+ leak. Indeed, mitochondria can also be uncoupled by several types of K^+ channels, nonselective cationic channels, and the PTP that have been reported in the IMM (99). Possible contributions of these channels are often overlooked in the respiratory analysis. Finally, compromised IMM integrity resulting from the isolation procedure is another factor that can contribute to basal respiration in suspensions of isolated mitochondria. Unfortunately, respiration experiments cannot reliably distinguish between these various pathways to clearly elucidate the mechanism of the basal respiration.

The existence of the FA-independent basal H^+ leak via UCP1 has been challenged by the groups of Martin Klingenberg and Keith Garlid (20, 21). These two groups performed experiments under more controlled conditions using UCP1 reconstituted in proteoliposomes. In this system, UCP1 transport activity can be isolated from that of other transport proteins, and the concentration of long-chain FAs can be better controlled owing to the lack of mitochondrial phospholipases or other enzymes that can supply UCP1 activators. These studies demonstrated that without long-chain FAs, there is no H^+ leak via UCP1 (20, 21). However, their use of artificial membranes to study UCP1 has been criticized by supporters of the basal H^+ leak.

Mitochondrial patch-clamp helped to address the problem of the basal H^+ leak in a rather precise manner. The first direct recording of the H^+ leak mediated by UCP1 (23) and AAC (22) across the native IMM confirmed the strict requirement of FAs. In these experiments, conditions such as the solution compositions on both sides of the IMM and the transmembrane voltage are well controlled. The H^+ leak is measured in isolation from other mitochondrial conductances and can also be easily distinguished from an unselective leak caused by a compromised membrane.

Importantly, extremely low concentrations of long-chain FAs can be achieved during whole-IMM patch-clamp recordings. Specifically, the recording chamber contains a single IMM (mitoplast) attached to the patch pipette, and the bath solution containing albumin or cyclodextrin is continuously perfused through the recording chamber. Albumin and cyclodextrins can also be added into the pipette (on the matrix side of the IMM). Therefore, a very limited amount of FA contained within a single IMM is exposed to almost an infinite amount of FA acceptors, bringing the FA concentration to zero. Under these conditions, no H^+ transport activity of UCP1 (8, 23) or AAC (22) is detected in the absence of FAs.

It should also be mentioned that the basal H^+ leak does not align well with the postulates of the chemiosmotic theory. An unregulated basal H^+ leak would render mitochondria permanently uncoupled and permanently inefficient for ATP production, while the chemiosmotic theory postulates that the H^+ permeability of the IMM should be tightly controlled. It would only make sense that such a fundamental physiological phenomenon as mitochondrial H^+ leak is precisely regulated, because ATP production and the metabolic integrity of the whole organism are at stake.

To conclude, the concept of the basal H^+ leak has been rightfully challenged based on evidence provided by direct mitochondrial patch-clamp measurements and reconstitution studies. Although the existence of the basal H^+ leak cannot be completely excluded, it must be realized that the experimental evidence in its support is weak.

Free Long-Chain Fatty Acids as Activators of Mitochondrial H^+ Leak

In the early 1950s, it was suggested that cells might contain a substance that uncouples oxidative phosphorylation, thus exerting control over cell metabolism. This theory was further extended to postulate that the uncoupling substance is not always present in an active form but is liberated from a precursor under certain conditions. This postulate was primarily based on the observation that freshly prepared liver mitochondria were not significantly uncoupled, but as they were incubated at room temperature, they developed many of the characteristics of freshly prepared mitochondria uncoupled with a protonophoric agent such as DNP (48, 100). The mysterious substance was later identified as a mixture of various nonesterified long-chain FAs (48, 100–102).

Later, in the 1960s, it was discovered that mitochondria isolated from brown fat behaved differently from mitochondria isolated from other tissues and were completely uncoupled immediately upon isolation (73, 103). Shortly after, a link between the uncoupled state of brown fat mitochondria and long-chain FAs was established (104, 105). The Nicholls group demonstrated that the action of FAs in brown fat mitochondria was associated with an increased H^+ conductance of IMM (74). Eventually, this H^+ conductance was shown to be mediated by a highly abundant protein of brown fat mitochondria, UCP1 (6, 9, 27).

In a parallel line of research, experiments with pigeons, seals, and rodents demonstrated that acute exposure to cold markedly increased uncoupling of skeletal muscle mitochondria isolated from these animals (reviewed in 70). This uncoupling was inhibited by the FA acceptor albumin and later demonstrated to be associated with an increased concentration of long-chain FAs (70, 106). This research eventually led to the identification of AAC as the protein that could at least partially explain the FA-dependent increase in H^+ leak across the IMM of skeletal muscle, heart, liver, and kidney (28, 29, 88, 89).

In addition to long-chain FA, other UCP1 activators were proposed, most notably long-chain acyl-CoA (107), coenzyme Q (108), and 4-hydroxy 2-nonenal (109), but their ability to activate UCP1 and AAC remained controversial (61, 110, 111). Patch-clamp recordings demonstrated that oleoyl-CoA strongly inhibited the UCP1-dependent H^+ leak instead of inducing it, whereas coenzyme Q6 had no effect (23). 4-Hydroxy 2-nonenal also showed no effect on UCP1 current, but it potentiated FA-dependent H^+ leak via AAC (22, 23). Importantly, 4-hydroxy 2-nonenal

could not increase the AAC-dependent H^+ leak without the presence of FAs (22). Thus, free long-chain FAs are the only established physiological activators of UCP1 and AAC.

In brown fat, it has been proposed that free FAs for UCP1 activation are generated via the hydrolysis of cytoplasmic lipid droplets (1, 2, 110). This process is under the control of the sympathetic nervous system that innervates brown adipocytes and activates adaptive thermogenesis mediated by these cells. In response to cold, diet, and perhaps other physiological stimuli, the sympathetic nervous system releases norepinephrine, which activates β3-adrenergic receptors on the surface of brown adipocytes, eventually leading to the hydrolysis of cytoplasmic lipid droplets (1). Free cytosolic long-chain FAs released via the hydrolysis of lipid droplets are postulated to serve both as UCP1 activators and the substrate for mitochondrial heat production (1, 2).

The assumption that H^+ leak via UCP1 is activated by free long-chain FAs derived from the hydrolysis of cytoplasmic lipid droplets was based, to a great extent, on in vitro assays in which exogenous FAs were infused into a suspension of isolated brown fat mitochondria (2, 112). In this experimental setting, UCP1-dependent uncoupling is indeed activated by extramitochondrial FAs. However, the role of free long-chain FAs generated by hydrolysis of cytosolic lipid droplets in UCP1 activation is less clear in intact brown adipocytes in vivo. The concentration of free long-chain FAs is tightly controlled via FA binding to proteins, esterification, and other modifications (113), rendering them unable to activate H^+ leak via UCP1/AAC. Mitochondria in particular have a sophisticated system for the modification and metabolism of free FAs (114), and thus, delivery of free FAs across the outer membrane to UCP1 located in the IMM could be challenging. Thus, free long-chain FAs generated within the IMM can have a comparative advantage for UCP1 activation as compared to those coming from the cytosol. Interestingly, direct patch-clamp studies of the UCP1-dependent H^+ leak in brown and beige fat mitochondria suggested that free FAs are locally produced via the hydrolysis of phospholipids of the IMM (8, 23). This indicates the existence of a protein endowed with a phospholipase activity (likely PLA2) in the IMM of brown fat. By hydrolyzing IMM phospholipids, this protein can strongly contribute to H^+ leak activation via UCP1 along with hydrolysis of cytosolic lipid droplets. Overall, the signaling pathway that connects the engagement of adrenergic receptors on the surface of brown adipocytes and UCP1 activation in the IMM could be more complex than is currently assumed.

The sources of free long-chain FAs for activation of H^+ leak via AAC in nonadipose tissues are even less explored. Because under normal conditions the density of cytosolic lipid droplets in such tissues is much less than that in brown fat, other mechanisms may play more significant roles. Thus, local membrane FA production could be an important mode of activation of H^+ leak via AAC. In this regard, PLA2 activity has been reported in not only mitochondria of brown/beige fat (8, 23) but also mitochondria of various nonadipose tissues. In fact, mitochondrial PLA2 activity was first identified as the reason why isolated liver mitochondria lose their capacity for coupled respiration as they age upon isolation (115, 116). Later, PLA2 activity was implicated in different physiological and pathophysiological aspects of mitochondrial function such as uncoupling and energy metabolism, remodeling of mitochondrial phospholipids to protect against oxidative damage, PTP activation, and cell death (96, 97, 117–126). It is suggested that Ca-independent PLA2 (iPLA2) is the predominant mitochondrial PLA2 in nonadipose tissues such as skeletal muscle, liver, heart, kidney, and brain (96–98, 117, 118, 121, 122, 124), but the actual identity of the enzyme responsible for the hydrolysis of mitochondrial phospholipids and generation of free long-chain FAs remains elusive. Also, it is possible that FA production is not mitochondrial but associated with remnants of other cellular organelles tethered to mitochondria after isolation, such as the endoplasmic reticulum or lipid droplets.

The physiological concentrations of extramitochondrial long-chain FAs needed to induce H^+ leak via UCP1 and AAC are still under debate, ranging broadly from low nanomolar to

high micromolar concentrations. Such discussion has only a limited applicability to those free FAs that are produced within the IMM. However, the question is still relevant when the source of free long-chain FAs is cytosolic. Early studies found that nanomolar FA concentrations (10–50 nM) are sufficient to activate H^+ leak via UCP1 in isolated brown fat mitochondria respiring in vitro (94). However, micromolar concentrations are required to activate H^+ leak via either UCP1 (127, 128) or AAC (129) functionally reconstituted in lipid bilayers. Direct patch-clamp analysis of UCP1 and AAC in the native IMM also demonstrated that low micromolar FA concentrations are needed to activate H^+ currents via these proteins (8, 22, 23). Finally, it was estimated that free cytosolic FAs can reach millimolar concentrations during active lipolysis in brown adipocytes (130), demonstrating that achieving micromolar concentrations of FA in the immediate proximity of UCP1 is not impossible physiologically.

With several groups finding that low micromolar concentrations of FAs are required for activation of H^+ leak via UCP1, why did the early results with isolated brown fat mitochondria suggest that the required FA concentrations were two orders of magnitude lower? One possible explanation is the use of the albumin buffer for free long-chain FAs in these studies (94). In these experiments, the ~60 μM bovine serum albumin buffer was mixed with tens to hundreds of micromoles of free palmitate to achieve nanomolar concentrations of free, unbound FAs (94). The calibration of this buffer was performed in the absence of isolated mitochondria. However, when this albumin-based FA buffer was mixed with a mitochondrial suspension, bound FAs were likely displaced from albumin by various lipids released from the mitochondrial membranes. Importantly, the FA-binding sites of albumin are not selective for FAs (131), and FAs can be displaced from albumin by many types of lipids. The situation is further exacerbated by continuous generation of free FAs and other lipids within the mitochondrial membranes, because these could eventually overwhelm the albumin buffer. One example is the mitochondrial PLA2 activity reported by several groups (8, 23, 96–98). Thus, the actual concentration of free FAs provided by the so-called FA albumin buffer will be significantly higher in the presence of mitochondria, potentially approaching the total concentration of FAs (hundreds of micromolars) added into the albumin buffer initially. Thus, use of the albumin buffer in respiration experiments could result in underestimation of the free FA concentration required to activate UCP1 by about two orders of magnitude (94). Such underestimation was also noted by others (21).

Direct patch-clamp analysis offers the best possible control of FA concentrations while studying UCPs in the native IMM. Only one microscopic vesicle of the whole IMM (mitoplast) is present in the 500-μL recording chamber. This single mitoplast does not affect the concentration of FAs in the large, continuously perfused recording chamber. In this experimental setting, the free FA concentration can be controlled directly by the simple addition of the required concentration. This electrophysiological analysis demonstrated that low micromolar concentrations of free long-chain FAs are required to activate H^+ currents via UCP1 (22, 23), supporting the data obtained with purified UCP1 and AAC incorporated in lipid bilayers (127–129).

To summarize, free long-chain FAs are the only established physiological activators of H^+ leak via UCP1 and AAC. Although we still cannot exclude the possibility that other activators of UCP1 and AAC exist within the cell, in order to activate H^+ leak via UCP1 and AAC, they may need to share certain chemical properties with FAs. These properties and the proposed mechanism by which FAs activate H^+ leak via UCP1 and AAC are discussed in the next section.

The Mechanism by Which Fatty Acids Activate H^+ Leak via UCP1 and AAC

UCP1 is the first and most-studied mitochondrial UCP. Almost everything we know about the mechanism by which long-chain FAs activate mitochondrial H^+ leak comes from studies of this

protein. Several different models of how UCP1 facilitates H^+ translocation in the presence of FAs have been proposed and are discussed in previous reviews (1, 77). These models vary significantly in two aspects: (*a*) the species transported by UCP1 (H^+ versus OH^- versus FA anions) and (*b*) the requirement of FAs for UCP1-dependent H^+ leak (required versus not required). These models were proposed based on indirect measurements of the UCP1-dependent H^+ leak, such as mitochondrial respiration or flux studies in proteoliposomes reconstituted with UCP1. When the method to directly measure UCP1-dependent H^+ leak using patch-clamp electrophysiology was introduced (23), it generated a trove of new data regarding the functional properties of UCP1. In particular, the high amplitude and time resolution of patch-clamp electrophysiology enabled the first measurement of transient currents generated by UCP1 in the presence of low-pK_a analogs of long-chain FAs. Because UCP1 does not translocate H^+ in the presence of such analogs, these transient currents revealed that UCP1 moves long-chain FAs within the membrane and undergoes a conformational change, as transporters do in the presence of a bona fide substrate. Another notable observation enabled by patch-clamp electrophysiology was a striking asymmetry of UCP1 in terms of its activation by cytosolic versus matrix long-chain FAs. Long-chain FAs failed to activate UCP1 currents when applied on the matrix side of the IMM but activated robust currents when applied on the cytosolic side (23).

The combination of all UCP1 functional properties revealed by patch-clamp electrophysiology could not be fully explained by any previous model of UCP1 function (23). Therefore, the need to develop a new model that incorporates all previous and new observations arose. UCP1 is a member of the SLC25 family of mitochondrial anion carriers. All members of this family are postulated to share a common mechanism of action (132). They all have a single substrate binding site located in the center of the membrane, which is alternatingly exposed to the opposite IMM sides through the conformational change of the carrier (**Figure 2*a***). There are two principal conformation states of SLC25 family members, the c-state with the substrate binding site exposed to the cytosol and the m-state with the substrate binding site exposed to the mitochondrial matrix. Based on the symmetry analysis of the amino acid residues at its putative substrate binding site, UCP1 was predicted to transport small carboxylic or keto acids in symport with protons (133). Transport of FAs by UCP1 was also postulated in the FA-cycling model of UCP1 function, though H^+ ions were assumed to be transported outside of UCP1 through a FA flip-flop across the lipid bilayer (20). When UCP1 currents were analyzed by patch-clamp electrophysiology, it was determined that UCP1 likely operates as a FA anion/H^+ symporter; however, this appeared to be true only for short-chain FAs (23). Short-chain FA anions could bind to UCP1 on both sides of the IMM and be transported to the opposite side. In contrast, long-chain FA anions could only bind on the cytosolic face of the IMM. Also, they appeared to anchor inside UCP1 and produced transient currents in response to changes in transmembrane voltage, as compared to steady currents generated by short-chain FAs (23). This anchoring was due to the longer carbon tails of the long-chain FAs and was therefore mediated by a hydrophobic interaction (23).

Therefore, from the viewpoint of the classical mechanism of SLC25 function, short-chain FAs could be considered bona fide, although low-affinity, UCP1 substrates. In contrast, long-chain FAs appear to hijack the UCP1 transport mechanism by lodging within the UCP1 substrate binding site with high affinity and becoming, de facto, continuously associated substrates. While continuously associated with the UCP1 substrate binding site, a single long-chain FA can facilitate multiple c–m conformation changes and help to transport multiple H^+ ions via UCP1 (**Figure 3*a***). Thus, in the presence of long-chain FAs, UCP1 operates like a H^+ uniporter (a transporter that has a single transport substrate). This mechanism of UCP1 function draws another analogy. The long-chain FAs lodged within UCP1 can be considered a cofactor for H^+ transport via UCP1. The Klingenberg group (21) first proposed a FA-cofactor model for UCP1 function,

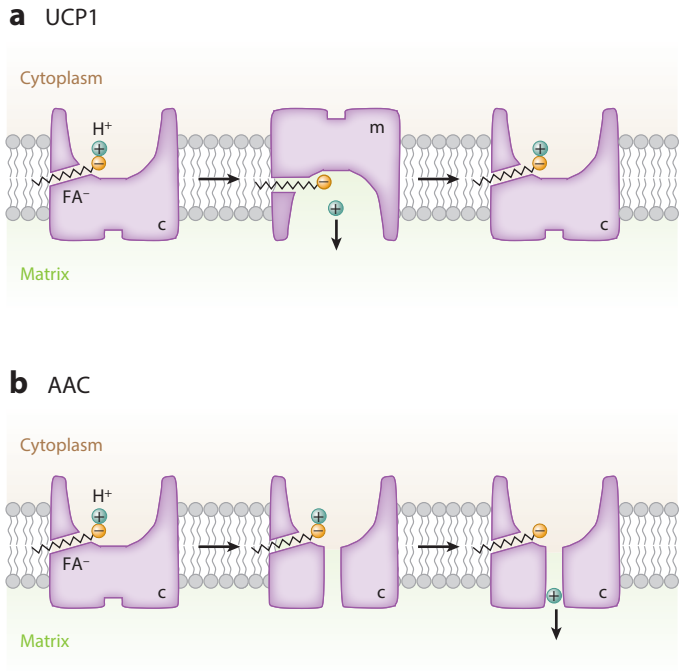

Figure 3

Mechanisms of FA-dependent H^+ transport by UCP1 and AAC. (*a*) The mechanism of H^+ leak via UCP1. UCP1 functions as a FA anion/H^+ symporter. FAs are UCP1 transport substrates and induce UCP1 transition between the c- and m-states. When long-chain FAs and H^+ are bound, UCP1 undergoes conformation changes between the c- and m-states, enabling H^+ translocation. However, long-chain FAs do not dissociate from UCP1 because they are caught within UCP1 by hydrophobic interactions. Thus, each long-chain FA can facilitate transport of multiple H^+. (*b*) The mechanism of H^+ leak via AAC. In contrast to UCP1, AAC does not transport FA anions, and FAs cannot induce AAC transition between the c- and m-states. Instead, binding of a long-chain FA anion to AAC might induce a minor conformational change to open a narrow translocation pathway for H^+ across AAC. In addition, long-chain FAs provide an essential binding site for H^+ permeation through AAC. Abbreviations: AAC, ADP/ATP carrier; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; FA, fatty acid; UCP1, uncoupling protein 1.

in which FAs bind within the UCP1 translocation pathway and its protonatable headgroup provides a missing "stepping stone" for H^+ transport via UCP1. The model presented in **Figure 3*a*** differs from their cofactor model in that FAs are rather unusual cofactors capable of inducing a large conformational change within the apoprotein (UCP1). This conformational change is likely required for successful H^+ translocation (23).

What are the structural features of long-chain FAs that help them to bind within UCP1 and activate H^+ leak? As mentioned earlier, the length of the carbon tail is the crucial factor (21, 23), which is likely explained by the hydrophobic interactions established by FAs when they bind to UCP1. There might be a hydrophobic pocket within UCP1 that accommodates the carbon tail of FAs, as illustrated in **Figure 3*a***, or the carbon tail may protrude into the lipid bilayer, while the polar head of FAs resides within UCP1. The degree of saturation of the carbon tail is not a major actor in H^+ leak activation (21, 23). Finally, a protonatable polar head plays a crucial role in activation of UCP1 by FAs. This is because it likely participates directly in the binding

and translocation of H^+. Non-protonatable low-pK_a FA analogs (such as alkyl sulfonates or halogenated FAs) do not activate UCP1 and competitively inhibit H^+ leak activated by regular FA (23, 77, 134). It must be kept in mind that although FAs in solution have a pK_a around 4.8, the majority of membrane-bound FAs are protonated at physiological pH, as their pK_a can increase to ~7 once they are incorporated into a phospholipid membrane (135, 136). In contrast, low-pK_a FA analogs such as alkyl sulfonates (solution p$K_a \approx -2$) stay unprotonated at physiological pH even after incorporation into a membrane (137).

The mechanism of activation of H^+ leak via AAC appears to be overall similar to that of UCP1 (22). First, AAC does not appear to conduct H^+ in the absence of FAs. Second, long-chain free FAs activate H^+ leak via AAC, and their potency depends on the length of the carbon tail. Third, the capacity of FAs to bind H^+ at physiological pH is essential for activation of H^+ leak via AAC, and low-pK_a FA analogs induce no H^+ current via AAC. Finally, FAs activate H^+ leak only when added on the cytosolic side of the IMM and not when added on the matrix side (22). Therefore, the presence of protonatable FAs within the translocation pathway, enabled by hydrophobic interactions, appears to be the common criterion for H^+ transport by UCP1 and AAC (22).

Interestingly, FAs could not induce the c–m conformational change of AAC, as probed with the c-state-specific AAC inhibitor CATR (22). This supports the concept that FAs are not transport species of AAC, contrary to adenine nucleotides that induce the AAC c–m conformational change (22, 31, 138). Despite the inability of FAs to induce the c–m conformational change in AAC, negatively charged low-pK_a FA analogs induce low-amplitude, AAC-dependent transient currents that are indicative of a small conformational change that FA anions can facilitate in AAC (22). Thus, a likely mechanism by which long-chain FAs activate H^+ leak via AAC is that of a cofactor for H^+ translocation (**Figure 3*b***). Binding of a long-chain FA anion within AAC can enable H^+ translocation by providing a missing binding site in the H^+ translocation pathway and facilitating a small conformational change within AAC that may open a narrow H^+ translocation pathway through the closed gate of AAC. To activate H^+ leak via AAC, long-chain FAs must bind from the cytosolic face of the IMM, but AAC can be in either the c- or m-state (22) (**Figure 2*b***).

Regulation of Mitochondrial H^+ Leak by Purine Nucleotides

Whereas FAs are the principal physiological activators of H^+ leak via UCP1 and AAC, Mg^{2+}-free purine nucleotides are the main physiological inhibitors. Thus, the amount of H^+ leak via UCP1 and AAC under physiological conditions is the result of a fine balance between activation by FAs and inhibition by purine nucleotides. Despite some similarities, the mechanisms by which nucleotides inhibit H^+ leak via UCP1 and AAC are not identical.

UCP1 and AAC possess binding sites that recognize only Mg^{2+}-free purine nucleotides. However, these binding sites do not have the same nucleotide specificity. AAC binds ADP and ATP with an affinity about 10 times greater than that for GDP and GTP. In contrast, the affinities of UCP1 for adenine and guanyl nucleotides are about equal (31, 76). As GDP does not bind to AAC, it is often used in biochemical assays as a UCP1-specific ligand. Despite the ability of UCP1 to bind GDP and GTP, ADP and especially ATP are likely to be its principal physiological inhibitors in vivo because of their abundance.

The nucleotide-binding site for UCP1 is on the cytosolic face of the IMM, whereas that of AAC opens alternatively to both sides of the IMM (31, 76). This is because nucleotides are inhibitors of UCP1, whereas they are transport species of AAC. Moreover, nucleotides first bind to AAC with low affinity, but as AAC undergoes the c–m conformational change required for transport, the affinity of AAC for nucleotide binding increases in the transition state, in accordance with the induced transition fit of the transport catalysis (31, 76). In contrast, UCP1 binds nucleotides

with higher affinity and does not undergo the nucleotide-induced transition fit, because it does not transport nucleotides (31, 76).

Nucleotide regulation is particularly well characterized for UCP1. In brown adipocytes, UCP1 is tonically inhibited by purine nucleotides, primarily ATP. Binding of purine nucleotides on the cytosolic side of UCP1 obstructs the UCP1 translocation pathway for H^+ (76). To activate brown fat thermogenesis, FAs must overcome this inhibition. However, whether FAs remove purine nucleotide inhibition by direct competition or another mechanism has remained controversial (127, 139–142). Direct electrophysiological analysis of UCP1-dependent H^+ current helped to demonstrate competition between FAs and purine nucleotides for binding to UCP1 (23, 77). Structurally, FA anions and ATP are very different and unlikely to bind to the same site. However, the binding sites for FA anions and ATP may partially overlap or be located in immediate proximity, so that electrostatic repulsion between the two negatively charged molecules results in competition.

In contrast to the well-characterized purine nucleotide regulation of H^+ leak via UCP1, little was known about the mechanism by which nucleotides regulate H^+ leak via AAC. The recent application of the patch-clamp technique to directly record both the ADP/ATP exchange current and the FA-induced H^+ leak mediated by AAC was instrumental in understanding the mechanism of their interaction (22). Remarkably, active nucleotide exchange significantly inhibits FA-dependent H^+ leak but does not suppress it completely. This is not very surprising, because in contrast to their action in UCP1, nucleotides are not inhibitors of AAC but its transported species. Adenine nucleotide exchange and FA-induced H^+ leak occur through the AAC translocation pathway and interfere with each other. However, the affinities with which FAs and adenine nucleotides bind to AAC are comparable, and they cannot completely inhibit each other at their physiologically relevant concentrations (22).

The competition of nucleotide exchange and FA-dependent H^+ leak in the AAC translocation pathway suggests intimate interconnection between mitochondrial ATP and heat production. By combining two transport modes, one controlling mitochondrial ATP production and another controlling mitochondrial thermogenesis, AAC can set the ratio between these two forms of mitochondrial energy output (**Figure 4**).

Regulation of H^+ Leak by Mitochondrial Hyperpolarization and Oxidative Conditions

In addition to its role in thermogenesis, the mitochondrial H^+ leak is an important regulator of mitochondrial production of reactive oxygen species (ROS). H^+ leak reduces mitochondrial ROS production (19, 143) and has been implicated in preventing oxidative damage to mitochondria and cells, as well as in promoting longevity (144, 145).

Mitochondria are the primary source of ROS in the cell and contain several sites of ROS production, notably the ETC and particular enzymes for substrate catabolism in the mitochondrial matrix (146). ROS production in suspensions of isolated mitochondria is sharply increased when $\Delta\Psi$ is hyperpolarized above a certain threshold (143). This is because high $\Delta\Psi$ makes it more difficult for the ETC to pump H^+, also slowing electron transport via the ETC. The slow electron transport leads to saturation of the electron carrier sites in the ETC and the upstream mitochondrial substrate catabolism pathways, causing the premature leak of the electrons to oxygen and excessive ROS production (146).

Mitochondria were proposed to possess a mechanism that prevents ROS generation caused by $\Delta\Psi$ hyperpolarization. Specifically, the mitochondrial H^+ leak was postulated to be nonohmic; i.e., it increases sharply when $\Delta\Psi$ becomes very negative (143). Such a sharp increase in H^+ leak would depolarize $\Delta\Psi$, preventing excessive ROS production. This could be useful under

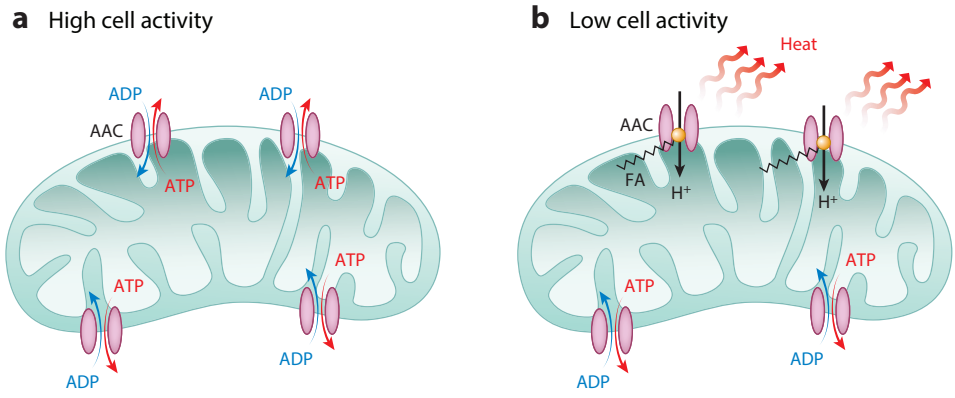

Figure 4

The AAC regulates the balance between mitochondrial ATP and heat production. (*a*) During periods of high cell activity, such as in working skeletal muscle, the requirement for ATP production is high, and the majority of AAC molecules are involved in ADP/ATP exchange. Under these conditions, mitochondrial thermogenesis is low. (*b*) When cell activity is low, such as in resting skeletal muscle, AAC molecules are less occupied by ADP/ATP exchange. Under these conditions, long-chain FAs can interact with more AAC molecules, generating H^+ leak and activating thermogenesis. Abbreviations: AAC, ADP/ATP carrier; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; FA, fatty acid.

conditions when a cell undergoes a fast transition from a high to low level of activity. As an example, in skeletal muscle transitioning from high activity to rest, $\Delta\Psi$ could hyperpolarize because of the reduction in ATP consumption, leading to increased ROS production (143). However, the nonohmic H^+ leak prevents such $\Delta\Psi$ hyperpolarization and associated ROS production. When the AAC-dependent H^+ leak was recorded with patch-clamp electrophysiology, its sharp increase with $\Delta\Psi$ hyperpolarization was confirmed (22). The H^+ leak via UCP1 is also nonohmic but to a lesser degree than that via AAC (23).

The early respiration studies also suggested that mitochondrial H^+ leak is upregulated in the presence of superoxide, proposing that ROS potentiate mitochondrial H^+ leak directly (147). This highlighted the second important negative feedback mechanism for the regulation of mitochondrial ROS production and indicated that UCPs should be potentiated by ROS. After UCP2 and UCP3 were identified, ROS activation of the mitochondrial H^+ leak was first assigned to them (148). However, when the mitochondrial H^+ leak was measured directly with the patch-clamp electrophysiology, it was determined that AAC, and not UCP2 or UCP3, is responsible for the potentiation of H^+ leak under oxidative conditions (22). This oxidative potentiation of the AAC-dependent H^+ leak is strictly dependent on prior activation by FAs, which eliminates the possibility of any FA-independent, parallel mechanism of H^+ leak activated by oxidation (22). Redox modifications of AAC cysteine residues have been suggested to regulate ADP/ATP exchange (31, 149). Similarly, potentiation of the FA-dependent H^+ leak via AAC under oxidative conditions could be due to modification of cysteine residues, as was also recently reported for H^+ leak mediated by UCP1 in brown fat (71, 150).

DISCLOSURE STATEMENT

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