Review

Lipotoxicity, fatty acid uncoupling and mitochondrial carrier function

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ABSTRACT

Diseases like obesity, diabetes or generalized lipodystrophy cause a chronic elevation of circulating fatty acids that can become cytotoxic, a condition known as lipotoxicity. Fatty acids cause oxidative stress and alterations in mitochondrial structure and function. The uncoupling of the oxidative phosphorylation is one of the most recognized deleterious fatty acid effects and several metabolite transporters are known to mediate in their action. The fatty acid interaction with the carriers leads to membrane depolarization and/or the conversion of the carrier into a pore. The result is the opening of the permeability transition pore and the initiation of apoptosis. Unlike the other members of the mitochondrial carrier superfamily, the eutherian uncoupling protein UCP1 has evolved to achieve its heat-generating capacity in the physiological context provided by the brown adipocyte and therefore it is activated by the low fatty acid concentrations generated by the noradrenaline-stimulated lipolysis.

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1. Fatty acids as cytotoxic agents

Long-chain non-esterified fatty acids (FA), also referred to as free fatty acids, are an important energy source for most body tissues, particularly during periods of starvation or prolonged endurance exercise. Under physiological conditions, FA are not really "free" since they are avidly bound by proteins like albumin in the circulation or fatty acid binding proteins within cells. FA are more than substrates for oxidative metabolism and thus they play a significant role in cell signalling, influence enzymatic activities, gene expression, ion homeostasis, etc. While fluctuations in the concentration of circulating FA are important for the physiological regulation of a number of processes, sustained high FA levels are deleterious [1,2]. Thus, diseases like obesity, diabetes or generalized lipodystrophy cause a chronic elevation of circulating FA that can result in cytotoxic intracellular levels. This condition, termed "lipotoxicity", can lead to the apoptotic death of the cell which is then called "lipoapoptosis" [2]. The lipotoxic action is dependent on the chain length and saturation of the FA molecule. Unsaturated FA are less toxic than their saturated counterparts and can even attenuate the toxicity of the latter [3,4].

The investigation of the molecular mechanisms behind the FA-induced lipotoxicity has mainly analyzed the mitochondrial effects. However, it must be borne in mind that FA can also affect other sites and, for example, they cause endoplasmic reticulum stress [5] or increase the production of superoxide by the NADPH oxidase [6] (Fig. 1). The mitochondrial effects are of special relevance because this organelle plays a key role in the initiation of apoptosis. The intermembrane space confines a number of proapoptotic factors (AIF, smac-DIABLO or cytochrome *c*, etc.) that, when released, initiate a caspase-dependent cell death cascade [7]. The release can be induced either by a direct permeabilization of the outer mitochondrial membrane (OMM) or by a change in the permeability properties of the inner mitochondrial membrane (IMM). The apoptosis-related alteration of the permeability properties of the IMM has been termed "permeability transition" and is due to the opening of a pore, the permeability transition pore (PTP), that allows the permeation of solutes of up to 1500 Da. Opening of this pore causes the collapse of membrane potential, matrix swelling and culminates with the rupture of the OMM [7,8]. The PTP is a complex whose composition is dynamically regulated by a variety of stimuli and conditions. It includes cytosolic proteins and proteins from the OMM and IMM. The most prominent components are the voltage-dependent anion channel, hexokinase, creatine kinase, cyclophilin D and probably the phosphate carrier (PIC), the adenine nucleotide translocator (ANT) or other members of the mitochondrial transporter superfamily [9]. The presence of cyclophilin D confers the PTP its characteristic sensitivity to the inhibition by cyclosporin A. The permeabilization of the OMM can occur after the insertion and oligomerization of pro-apoptotic Bcl-2 family members (Bax, Bak, tBid) or the formation of ceramide

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Abbreviations: FA, fatty acid; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; PTP, permeability transition pore; PIC, phosphate carrier; ANT, adenine nucleotide translocator; ROS, reactive oxygen species; UCP, uncoupling protein; CPT-1, carnitine palmitoyl transferase-1; BAT, brown adipose tissue

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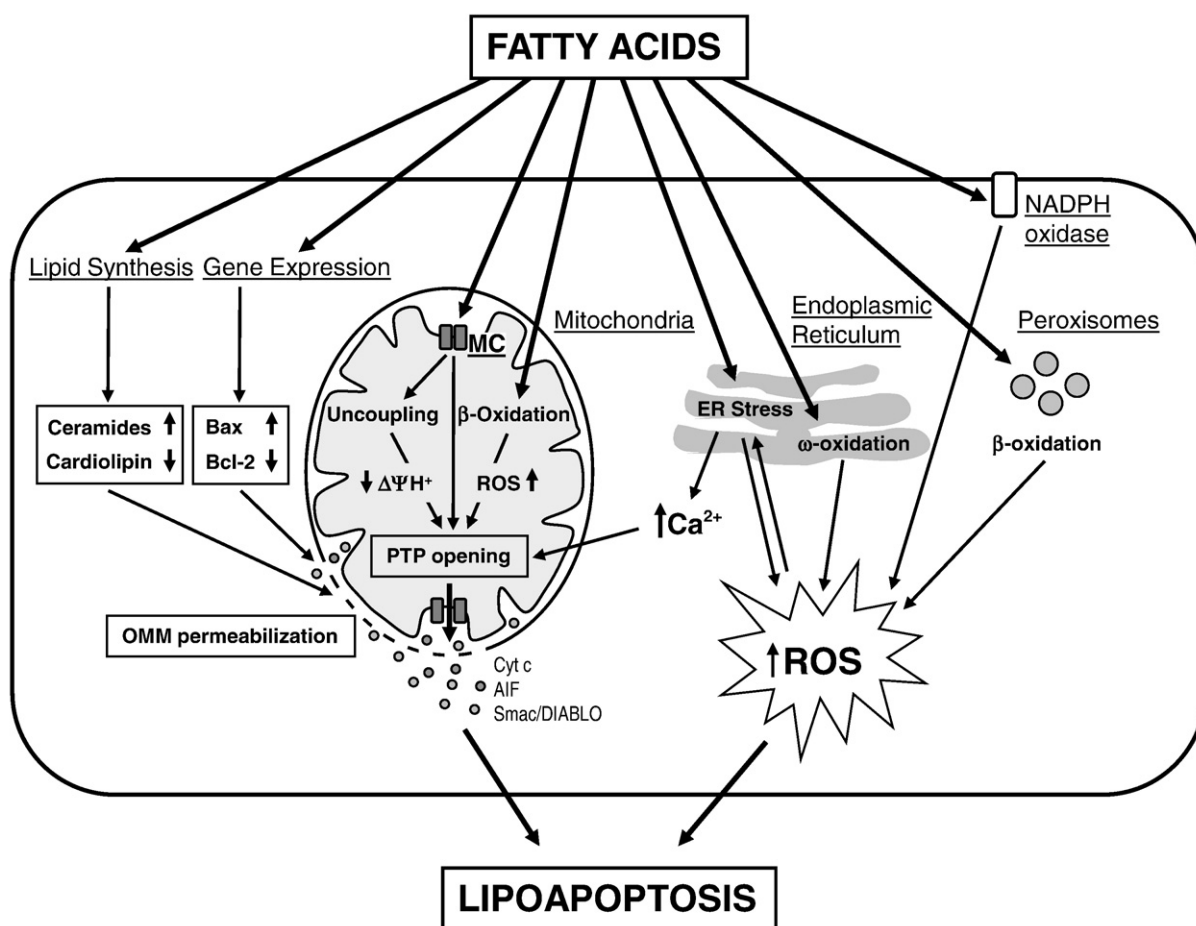 *E-mail address:* rial@cib.csic.es (E. Rial).

Fig. 1. Lipotoxic effects of fatty acids. FA affect the cell viability by altering mitochondrial function and generating oxidative stress. The oxidation of FA in mitochondria, peroxisomes (β-oxidation) and endoplasmic reticulum (ω-oxidation) generate ROS. FA also increase ROS production by the NADPH oxidase and give rise to endoplasmic reticulum stress. FA can cause the release of apoptogenic factors from mitochondria either by inducing the permeabilization of the OMM or by triggering the opening of the PTP. All these actions result in the initiation of the apoptotic cascade. "MC" represents any member of the mitochondrial carrier family. See text for further details.

aggregates both of which generate large pores in the membrane that allow the release of the apoptogenic factors [7,10].

The FA effects on mitochondria can be divided in three classes that are interrelated: (1) increase in the production of reactive oxygen species (ROS), (2) increase in mitochondrial proton conductance (uncoupling) and (3) opening of the PTP (Fig. 1). Thus, FA oxidation can cause an elevation of ROS and trigger PTP opening [6,11]. Additionally, FA can provoke the permeabilization of the OMM in two ways. Firstly, because saturated FA are precursors for *de novo* synthesis of ceramides and, secondly, because they up-regulate the expression of the pro-apoptotic Bax together with the down-regulation of the anti-apoptotic Bcl-2 [12,13]. Nevertheless, the uncoupling of the mitochondrial oxidative phosphorylation is one of the most recognized deleterious FA effects. The uncoupling phenomenon was first described by Pressman and Lardy in 1952 when they reported that FA could stimulate respiration in the absence of a phosphate acceptor and could also increase the ATPase activity [14,15]. For a long time it was assumed that FA would exert a conventional protonophoric action on the mitochondrial membranes, i.e. acting like classical uncouplers [16]. The uncoupling activity was explained with a cycle that required the transbilayer movement (flip-flop) of the protonated FA from the cytosolic to the matrix face of the inner membrane and the subsequent return of the anionic form to the cytosolic side. This cycle would result in the net delivery of protons to the matrix side. While the flip-flop of the protonated form is extremely fast, the movement of the anion is too slow and thus the mechanism of uncoupling was not satisfactory. In 1988, Skulachev and coworkers [17,18] showed that carboxyatractylate, an inhibitor of the ANT, could partially inhibit the

uncoupling effect of palmitate in liver mitochondria and proposed that the ANT was facilitating the translocation of the FA anion and thus providing the missing arm to the protonophoric cycle. Since then, several other mitochondrial carriers have been shown to mediate in the uncoupling effect of FA [3,19–21].

2. The mitochondrial carrier protein superfamily and the uncoupling protein family

The metabolite transporters of the IMM form a protein superfamily whose most distinctive feature is the internally repetitive structure where a sequence unit of 100 amino acids is repeated three times [22]. Each repeat contains two transmembrane segments linked by a long hydrophilic loop. These three loops are oriented toward the matrix side of the inner membrane, and include the conserved sequence motif that is currently used to identify potential members of the superfamily (NCBI conserved domain Pfam00153, mito_carr superfamily). The elucidation of the three-dimensional structure of the ANT has confirmed this structural arrangement [23]. Therefore, it appears that the superfamily evolved by triplication of a primordial protein that contained two transmembrane domains [24,25]. Moreover, since mitochondrial carriers do not appear to have orthologs in prokaryotes, it has been proposed that the ancestral mitochondrial carrier may have been an evolutionary innovation of the ancestral organism, which succeeded to host the bacterial endosymbiont that eventually became a mitochondrion [25,26]. The subsequent diversification generated the carrier

superfamily that ensures the highly dynamic traffic required for the integration of the mitochondrion in the cellular metabolism.

The uncoupling proteins (UCPs) are members of the superfamily whose biological function should, in principle, be to allow a regulated discharge of the proton gradient [27,28]. Therefore a UCP would dissipate energy although the actual molecular mechanism could vary among the different family members. In mammals five genes encode proteins considered UCPs [27,29]. UCP1 is present in brown adipose tissue (BAT), UCP2 is ubiquitous while UCP3 is expressed in skeletal muscle, heart and BAT. UCP4 and BMCP1 (UCP5) are predominantly expressed in the nervous system and are only distantly related to the rest of the family. Genes homologous to the mammalian UCPs have been described not only in most phyla of the animal kingdom but also in plants and unicellular eukaryotes [27,29]. It must be stressed, however, that the transport activity of most members of the UCP family has been inferred from their homology to UCP1 and that reports on their uncoupling activity are either lacking or controversial.

The function of the different members of the UCP protein family is not fully established but available data point to a general role in the protection against oxidative stress. Although the acceleration of respiration due to UCP-mediated uncoupling would lead to a reduction in the mitochondrial production of superoxide, it has also been proposed that UCPs induce a metabolic shift that promotes glycolysis and therefore indirectly lowers ROS production [30]. Nevertheless, there are many examples where UCPs are upregulated in physiological situations where there is oxidative stress and data showing that their overexpression reduces ROS damage. Thus, UCP2 is induced by agents that cause oxidative stress like lipopolysaccharide [31], TNFα [32,33] or fatty acids [34], or pathological conditions involving high ROS levels like liver injury [35], obesity [36], cancer [37] or diabetes [38–40]. Similarly, transgenic plants with increased UCP levels show higher tolerance to oxidative stress and UCP genes are upregulated when plants are exposed to oxidative stress generating agents [41]. All these observations have led to the consideration of the UCPs as part of the antioxidant defence system.

The eutherian UCP1 is, however, a protein with a distinct physiological function: heat generation in BAT, a tissue only found in eutherian mammals. This energy dissipatory mechanism is used by mammals to maintain their body temperature when cold exposed or to burn excess calories ingested with the diet. Additionally, eutherian UCP1 present two distinct biochemical properties: a high nucleotide-sensitive basal proton conductance and, as it will be later discussed, a high affinity for fatty acids (physiological activators) [42,43]. Recent phylogenetic analysis based on vertebrate UCP protein sequences and the reported conservation of syntenic regions have demonstrated that there are orthologs of UCP1 in mammals, amphibians, and fish [29,44–47]. The long branch leading to eutherian UCP1 is indicative of strong structural divergence although the observed amino acid changes are due to purifying rather than positive selection (Fig. 2) [45,46]. Hence, it seems clear that structural divergence was accompanied by a functional shift. It can be envisaged that ancestral UCP1 probably had a role in protection against oxidative stress in tissues where it was expressed, and that the coexistence of paralogs (UCP2 and 3) that could fulfill its function, together with the restriction of UCP1 expression to BAT allowed the functional co-option to assume the thermogenic role in eutherians [29,46]. Indeed, the carp UCP1 does not have a thermoregulatory function since its expression in the liver decreases when fish are exposed to cold and, furthermore, its functional characterization has demonstrated that it does not display the distinctive nucleotide-sensitive basal proton conductance found in the eutherian UCP1 [48].

3. Fatty acid uncoupling mediated by the mitochondrial carriers

As we have said earlier, the ability of FA to uncouple mitochondrial respiration has been known for decades although only recently it has been shown that the members of the mitochondrial carrier family participate in the protonophoric cycle. These observations have been made both with isolated mitochondria from different sources and also in reconstituted systems. The prominent inhibitory effect of carboxyatractylate is probably due to the high abundance of the ANT in the IMM. However, BAT mitochondria present a much higher sensitivity to FA uncoupling and, in fact, the first bioenergetic studies performed with these mitochondria clearly established the need for the presence of albumin in the respiration buffers to observe respiratory control [42,49]. This high FA sensitivity was subsequently linked to the presence of the uncoupling protein UCP1. These observations were later confirmed with the comparison of BAT mitochondria from cold-adapted animals with those reared at thermoneutral temperature where the FA effects correlated with the UCP1 content [50]. Subsequently, with the development of systems for the recombinant expression of the UCPs in yeasts, it has been possible to demonstrate that when UCP1 is expressed, *Saccharomyces cerevisiae* mitochondria display a FA sensitivity comparable to those from BAT [51]. Finally, ablation of UCP1 in transgenic animals results in a lower FA sensitivity [52]. Since this high affinity is not observed with mitochondria containing either UCP2 or UCP3, several groups have generated protein chimeras made of domains from the uncoupling proteins UCP1, UCP2 and UCP3 and demonstrated that the hydrophilic loop that connects the transmembrane domains 3 and 4 confers UCP1 its high FA affinity [53–55]. We have long emphasized that the high FA-affinity of UCP1 correlates with the physiological regulation of its thermogenic activity. Under non-thermogenic conditions, purine nucleotides maintain the protein inhibited and when noradrenaline signals the initiation of thermogenesis, its binding to β3-receptors sets off a lipolytic cascade. The FFA liberated serve two functions: they are the substrates for respiration and activators of UCP1 [42,43]. As we have already stated, the shift in the biochemical properties of this mitochondrial carrier fits with the regulation of thermogenesis, and provide evidence that eutherian UCP1 evolved to achieve its heat-generating capacity in the physiological context provided by the brown adipocyte [29]. The carrier-mediated FA-induced uncoupling has also been suggested to play a role in thermoregulatory heat production [16,18] and in the control of the generation of ROS [56].

A recent publication has shown that *Yarrowia lipolytica* does not have orthologs of the known members of the UCP family but that the oxaloacetate carrier (OAC) presents an uncoupling activity stimulated by FA and inhibited by purine nucleotides that resembles the activity and regulation of UCP1 [57]. *Y. lipolytica* is an oleaginous yeast with a dynamic FA metabolism that stores large amounts of triglycerides in lipid bodies [58]. In the exponential phase lipids are predominantly used to build membranes, while in the stationary phase, FA are used as carbon sources [59,60]. The FA activation of the OAC would be linked to the decrease in ROS production and/or the reoxidation of NAD(P)H under the conditions of low ATP demand of the stationary phase. We can envisage that the OAC in *Y. lipolytica* evolved to allow an FA-induced uncoupling activity while retaining the original transport activity [57]. This evolutionary adaptation would be linked to the peculiar metabolism of this oleaginous yeast and, for example, *S. cerevisiae* mitochondria show no evidence for the presence of a similar UCP-like activity [51,61] despite the presence of an ortholog of the Yarrowia OAC.

4. Mechanism of fatty acid action

The members of the mitochondrial transporter superfamily are mainly anion carriers. The experiments with BAT mitochondria in the early seventies indicated that these mitochondria had an atypical permeability to anions that was soon related to the nucleotide-sensitive uncoupling pathway responsible for the thermogenic capacity of the tissue [62–64]. However, the relationship between the anion permeability, high FA sensitivity and the energy dissipation pathway (UCP1) has been controversial. The fact that protons and anions share a common pathway has rarely been disputed due to the unequivocal demonstration of the competition between chloride and protons for the path and their identical nucleotide sensitivity [43,62,63]. However, the puzzling observation of the lack of influence of albumin, i.e. low FA
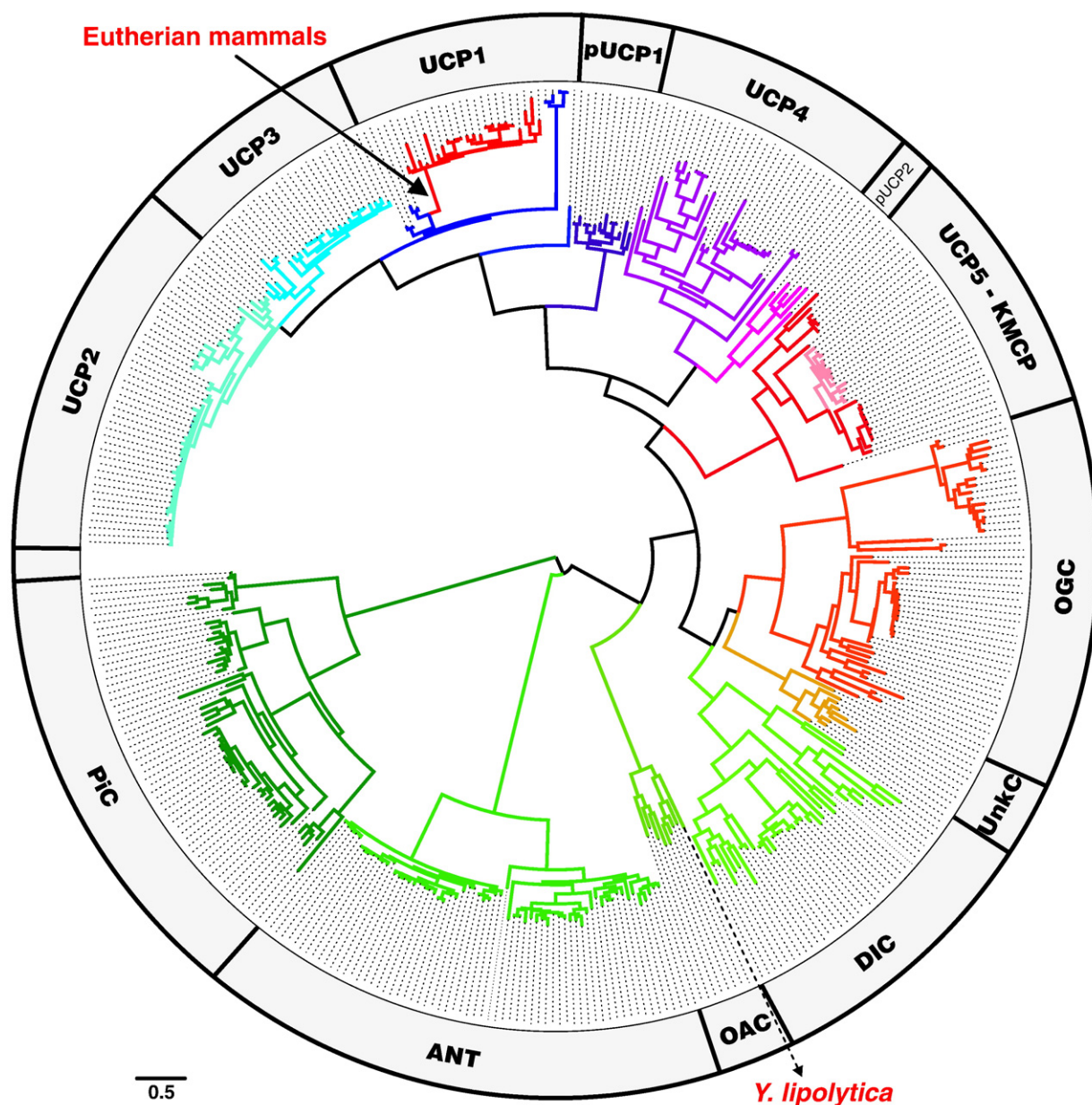
Fig. 2. Phylogenetic relationships of the uncoupling protein family and the mitochondrial carriers with highest homology. 400 full-length, non-redundant protein sequences corresponding to phosphate carriers (PIC); adenine nucleotide translocators (ANT); oxaloacetate carriers (OAC); dicarboxylate carriers (DIC); oxoglutarate carriers (OGC); uncoupling proteins (UCP) and a set of carriers of yet undefined function (UnkC) were retrieved from NCBI and ENSEMBL public databases and aligned. The maximum likelihood tree was inferred using PhyML. Tree topology was drawn by using Dendroscope and the circular tree generated with FigTree. Other abbreviations: BMCP1, brain mitochondrial carrier protein 1 (also termed UCP5); KMCP, kidney mitochondrial carrier protein; pUCP, plant uncoupling protein. The branch leading to the eutherian UCP1 and the position of the OAC from *Y. lipolytica* are indicated. See reference [57] for further details.

concentrations, on the chloride permeability and the demonstration that FA were the physiological regulators of proton transport through UCP1 were not easily reconciled. A hypothesis was put forward suggesting the existence of two pathways inside the protein, one FA-sensitive for protons and the other for anions such as chloride [63,64]. The latter demonstration that the reconstituted UCP1 could catalyze a nucleotide-sensitive transport of a wider variety of anions and that the transport rate increased with the anion hydrophobicity led to the proposal that FA were UCP1 substrates [65]. By that time, the involvement of the ANT in the uncoupling action of FA had been demonstrated and thus a common mechanism for the carrier-mediated FA-uncoupling was proposed [18]. The mechanism, known as the "fatty acid cycling hypothesis,", proposed that UCP1, like the rest of mitochondrial carriers, would catalyze the translocation of the anionic form of the FA and once in the cytosolic side of the membrane, the

carboxylate group would pick up a proton and the protonated FA would flip-flop back to the matrix side where the proton would be released and the protonophoric cycle completed (Fig. 3a) [18].

The mechanism of activation of UCP1 has been studied in detail over the years and the analysis of chemical nature of the compounds that increase the proton conductance has provided valuable information. The structural requirements for the active molecules do not appear to be very stringent, thus compounds with a free carboxylate group and with sufficient lipid solubility will increase the proton conductance. A second polar group cannot be present unless it is close to the carboxylate [66,67]. The FA-cycling hypothesis imposes restrictions on the nature of the active compounds since their protonated forms have to flip-flop across the IMM. Thus it has been shown in liposomes that a bulky-planar structure, like a benzene ring, at the end of the aliphatic tail hampers its permeation across the membrane [68,69]. However, *all-trans* retinoic
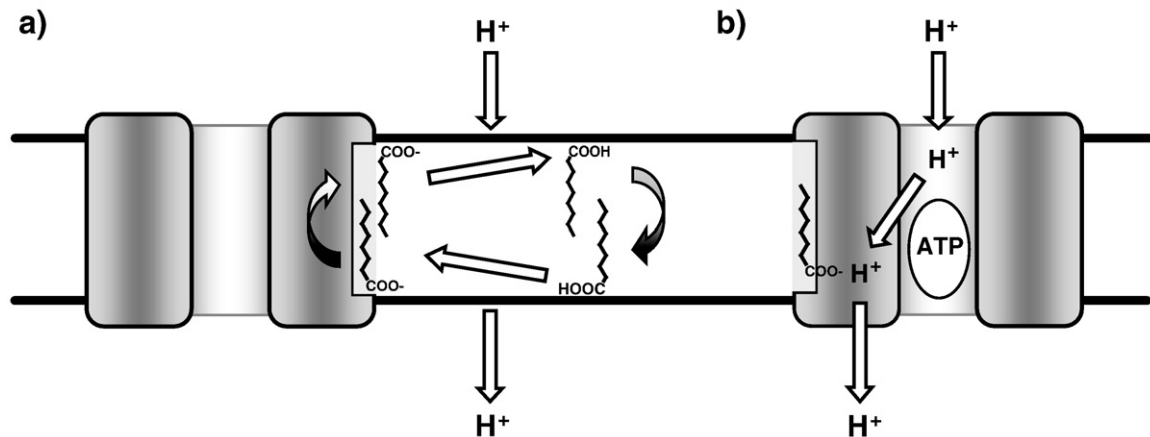
Fig. 3. Mechanisms for the activation of the uncoupling protein UCP1 by fatty acids. Two hypotheses have been put forward to explain the mechanism of activation. (a) "Fatty acid cycling hypothesis": UCP1 translocates the FA anion from the matrix to the intermembrane side of the IMM to complete a protonophoric cycle that allows a net flux of protons to the matrix. (b) "Proton buffering model": FA activate UCP1 overriding the nucleotide inhibition of the basal proton conductance. The carboxylate binds protons and delivers them to a site from which they are translocated to the matrix side of the membrane.

acid and a number of bulky retinoids are high-affinity UCP1 activators and, according to the liposome permeation data, they should not flip-flop [67]. Alkylsulfonates constitute another interesting group of compounds because of their resemblance to FA but with a pKa for the sulfonic group much lower than that of the carboxylate. The acid group cannot be protonated at physiological pH and therefore alkylsulfonates cannot act as a mobile proton carriers. Experiments in isolated mitochondria have demonstrated that long-chain alkylsulfonates do activate UCP1 and therefore they have become a fundamental argument against the "FA-cycling hypothesis" [20]. A subsequent paper questioned the undecanosulfonate data arguing that their uncoupling effect was due to the displacement of FA bound to albumin [70]. The displaced FA would be responsible for the observed activation of UCP1. However, a close scrutiny of the data presented reveals that, in the absence of albumin, undecanosulfonate was indeed active. The apparent lower activity was due to an increased basal proton conductance due to the lack of albumin. There is an alternative mechanism for the activation of UCP1 by FA known as the "proton buffering model" that proposes that UCP1 is a proton transporter and the carboxylate of the FA would be part of the proton translocation pathway (Fig. 3b) [20,43,64].

The expression of the uncoupling protein UCP3 has been linked to FA metabolism. Thus, situations that lead to an increase in plasma FA, such as fasting or high-fat feeding, cause an increase in muscle UCP3 mRNA levels [71]. In the same direction points the experiments where the overexpression of lipoprotein lipase [72] or the inhibition of carnitine palmitoyl transferase-1 (CPT1) by etomoxir raises the intracellular FA levels and also those of UCP3 [73]. Finally, the presence of a PPARα response element in the UCP3 gene promoter also suggests that UCP3 function could be related to FA metabolism [74]. Several putative roles have been ascribed to UCP3 in FA handling. First, UCP3 could have a detoxification role, removing lipid peroxides from the matrix side of the IMM [75]. The idea is that since FA oxidation leads to superoxide formation and this occurs mainly at the matrix side of the IMM, matrix bound FA would be especially prone to lipid peroxidation. Highly reactive FA peroxides could accumulate in the IMM and to avoid damage to the mtDNA, UCP3 would translocate the FA peroxides to the outer face of the IMM. The charges present in the aliphatic chain would prevent their return to the matrix side when protonated. A second role would be to export FA to prevent their accumulation in the mitochondrial matrix when there is an increase in FA supply [76]. This situation could be reached if, for example, certain fatty acyl-CoAs were poorly oxidised by mitochondria. Acyl-CoA thioesterases would release the CoA moiety and UCP3 could then export the non metabolised FA. The same would occur if FA supply were to exceed the oxidation rate as when CPT1 is inhibited by etomoxir [73].

In this case, however, it is not clear how the FA return would be prevented since the electrochemical proton gradient would cause the protonation of the FA anion and would drive its flip-flop back to the matrix side. Harper and co-workers have recently demonstrated that muscle mitochondria from wild-type and UCP3 knock-out mice equally export palmitate and that UCP3 is not required for FA oxidation, thus ruling out the hypothesis of a FA-translocating activity [77]. Their new data point for a role of UCP3 in the protection against oxidative stress.

5. Concluding remarks

High FA levels have deleterious effects on cells and mitochondria is one of their targets. The effects are mainly related to oxidative stress although they can also affect the efficiency of the oxidative phosphorylation. Several mitochondrial carriers have been shown to mediate in the uncoupling action of FA although the mechanism is not fully established. It is worth mentioning that several members of the carrier family have been shown to display the ability to switch from a carrier mechanism to a channel or pore mode [21,78]. Thus, it has been shown that under patch clamp conditions the ANT, PIC and UCP1 display channel conductances that range from the 25 pS in the PIC to 600 pS in the ANT. Modification of critical cysteine residues also alters the transport properties and, for example, thiol reagents and oxidative stress modify sulfhydryl groups in the ANT that lead to an increased probability of PTP opening [9]. FA also induce the classical cyclosporin-sensitive permeability transition that could be the result of two mechanisms that are non mutually exclusive [3,79,80]. First, their uncoupling activity could cause membrane depolarization and, second, their physical interaction with the ANT or PIC could alter, for example, the voltage sensor and cause the shift to the pore mode. The end result would be PTP opening, OMM permeabilization, release of apoptogenic factors and the initiation of apoptosis.

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