Interaction of free fatty acids with mitochondria: Coupling, uncoupling and permeability transition

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Abstract
Long chain free fatty acids (FFA) exert, according to their actual concentration, different effects on the energy conserving system of mitochondria. Sub-micromolar concentrations of arachidonic acid (AA) rescue ΔpH-dependent depression of the proton pumping activity of the bc1 complex. This effect appears to be due to a direct interaction of AA with the proton-input mouth of the pump. At micromolar concentrations FFA increase the proton conductance of the inner membrane acting as protonophores. FFA can act as natural uncouplers, causing a mild uncoupling, which prevents reactive oxygen species production in the respiratory resting state. When Ca2+-loaded mitochondria are exposed to micromolar concentrations of FFA, the permeability of the inner membrane increases, resulting in matrix swelling, rupture of the outer membrane and release of intermembrane pro-apoptotic proteins. The characteristics of AA-induced swelling appear markedly different in mitochondria isolated from heart or liver. While in the latter it presents the canonical features of the classical permeability transition (PT), in heart mitochondria substantial differences are observed concerning CsA sensitivity, ΔΨ dependence, reversibility by BSA and specificity for the activating divalent cation. In heart mitochondria, the AA-dependent increase of the inner membrane permeability is affected by ANT ligands such as adenine nucleotides and atractyloside. AA apparently causes a Ca2+-mediated conversion of ANT from a translocator to a channel system. Upon diamide treatment of heart mitochondria, the Ca2+/AA-induced CsA insensitive channel is converted into the classical PT pore. The relevance of these observations in terms of tissue-specific components of the putative PTP and heart ischemic and post-ischemic process is discussed.

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1. Introduction
Although the major part of long chain fatty acids (FFA) is bound to fatty acid binding proteins or associated with cellular membranes, a small portion is free and can exert, according to the actual concentration, a series of effects on cellular membranes in general and on mitochondrial membranes and their constituents in particular.

FFA have been found to accumulate under various conditions including fasting, exhaustive exercise [1], ethanol abuse [2,3], cold stress [4] and obesity [5]. Diabetes [6] as well as several hereditary disorders, such as the cerebro-hepato-renal (Zellweger) syndrome and Refsum disease are characterised by accumulation of FFA. Plasma concentration of phytanic acid may increase to 1–5 mM in patients suffering from Refsum disease [7]. Furthermore, FFA concentration has been found to increase greatly in ischemic and post-ischemic brain and heart as well as in blood of patients following acute myocardial infarction [8–10]. Deregulation in the control of cellular FFA level can affect cell proliferation and survival [11], owing to involvement of FFA, and arachidonic acid in particular, in the induction of necrosis [12] as well as apoptosis [11,13,14]. It has been shown that arachidonic acid-selective cytosolic phospholipase A2 is crucial in the cytotoxic action of tumor necrosis factor-α [15].

A role of mitochondria in cell death is clearly established. Different mechanisms may be involved, including (i) inhibition and/or uncoupling of oxidative phosphorylation with consequent energy failure; (ii) generation of reactive oxygen species
as a consequence of the inhibition of the normal electron transfer activity; (iii) alteration of membrane (inner and outer) permeability and consequent release of proapoptotic proteins normally confined in the intermembrane space.

This paper will focus on the interaction of FFA, arachidonic acid (AA) in particular, with mitochondria. Submicromolar concentrations of AA have a direct effect on cytochrome complexes of the respiratory chain, favouring the coupling efficiency of their proton pumping activities. When mitochondria are exposed to micromolar concentrations of AA, an uncoupling effect is observed, which per se is not responsible for the alteration of the inner membrane permeability of heart mitochondria. AA appears to exert different effects on the inner membrane permeability in heart and liver mitochondria. The relevance of these observations with respect to the constitution and/or assembly of the putative permeability transition pore (PTP) components is discussed.

2. Coupling effect of FFA on the respiratory chain cytochrome complexes

It has been shown that the $H^+/e^-$ ratio for proton pumping in liposome-reconstituted bc1 complex is, at the respiratory steady state (valinomycin present), significantly lower than that measured under level flow conditions [16,17]. Evidence was also obtained showing that the transmembrane $\Delta pH$ causes decoupling of the proton pumping activity at the aerobic steady state, either in the reconstituted system or in the native membrane [17]. An inverse relationship between the $H^+/e^-$ ratio of proton pumping and transmembrane $\Delta pH$ was found. BSA and CCCP, which caused an increase or a decrease of the $\Delta pH$ respectively, led to an opposite effect on the $H^+/e^-$ ratio (Fig. 1A). The $\Delta pH$-depressed steady state proton pumping activity was found to be reactivated by arachidonic acid at submicromolar concentrations (0.1–0.5 nmol/mg protein) (Fig. 1B). The transmembrane $\Delta pH$ was unaffected under these conditions, showing that the effect of AA was unrelated to its uncoupling property. The AA-dependent recoupling effect observed in bc1 vesicles resembles the rescue phenomenon observed in D96N mutated bacteriorhodopsin, whose proton pump activity was fully reactivated by azide [18]. This weak acid turned out also successful in reactivating the $\Delta pH$ depressed proton pump activity in bc1 vesicles [19]. The water-soluble azide was required at much higher concentrations (two orders of magnitude higher as compared with the submicromolar for the AA effect). These observations suggest that a steady state alkaline pH in the inner aqueous phase can depress protonation of residue(s) at the input mouth of the pump. Arachidonic acid may facilitate protonation of these critical residues and/or the semiquinone/quinol couple at the catalytic coupling site, thus acting as a protein internal protonophore. Similar conclusions were drawn by Sharpe et al. [20] who showed that the proton pumping activity was lost after incubation of cytochrome oxidase proteoliposomes with BSA, but was restored upon incubation of BSA-depleted proteoliposomes with FFA. Furthermore, Fetter et al. [21] have shown that fatty acids stimulate the activity and restore respiratory control in D132A subunit 1 mutated R. sphaeroides cytochrome c oxidase. All together, these observations suggest a supportive, or even essential, role of fatty acids in the proton pump mechanism of the cytochrome complexes of the respiratory chain.

3. FFA as protonophores

Free fatty acids are generally recognised as uncouplers of oxidative phosphorylation. The addition of micromolar concentrations of FFA to respiring mitochondria causes, in fact, a drop in transmembrane potential, an increase in state 4 respiration, a decrease of the respiratory control ratio and of the ADP/O ratio. This uncoupling effect is due to a cyclic movement of undissociated fatty acids, with the release of protons into the alkaline matrix space and subsequent efflux of the fatty acid anions mediated by the adenine nucleotide translocase (ANT) [22,23]. This mechanism is, however, not specific for ANT since other closely related mitochondrial anion carriers have been shown to be involved,
4. FFA and mitochondrial membrane permeability

Mitochondria play an important role in apoptosis through mechanisms involving permeabilisation of either the outer or the inner membrane. In both cases, pro-apoptotic intermembrane proteins will be released, including the apoptosis-inducing factor (AIF), endonuclease G (Endo G), Smac-DIABLO and cytochrome c. Outer membrane permeabilisation may be induced by the formation of protein channels by Bax, Bad and BID, which are thought to be involved in restructuring membrane lipids and induction of channel formation [35]. Furthermore, oligomeric aggregates of ceramide molecules apparently form channels in the outer membrane through which intermembrane proteins are released in the cytosol [36,37].

Permeabilisation of the inner membrane would instead cause equilibration of small solutes across the membrane, leaving matrix proteins to exert an osmotic pressure. The consequent massive swelling would be responsible for outer membrane rupture and release of intermembrane proteins.

Evidence has been accumulated indicating that fatty acids are involved in permeabilisation of the inner membrane, through different mechanisms that will be considered here below.

4.1. Induction of the permeability transition pore (PTP)

PTP is a non-specific, large conductance channel whose closed-open transition is regulated by a number of factors including matrix Ca^{2+} and Pi, mitochondrial membrane potential, redox state of pyridine nucleotides and adenine nucleotides. The pore is a multiprotein complex located at the contact sites of the mitochondrial membranes and thought to be constituted mainly by the voltage-dependent anion channel (VDAC), ANT and cyclophilin D (Cyp-D). FFA interaction with isolated mitochondria has been reported to induce PTP opening, with consequent matrix swelling. Given the protonophoric property of FFA, the PTP inducing effect may be expected to be a consequence of the uncoupling activity of FFA, which may cause PT to initiate by decreasing ΔΨ below the gating potential [38]. This can be referred to as an FFA indirect mechanism on membrane permeability. It has, however, been suggested that FFA can induce PT by a direct mechanism [39], involving an interaction with pore components, ANT in particular [7,40,41]. In isolated mitochondria, the FFA inducing effect is in fact modulated by ANT ligands and adenine nucleotides [42–45]. Moreover, long chain fatty acids increase the permeability of ANT–VDAC–hexokinase complex reconstituted into phospholipid vesicles. The reconstituted complex appears to retain all the properties of the native PTP [46]. In spite of the evidence in favour of the involvement of ANT in the permeability transition (see also [44,47,48] for review), experiments on mitochondria isolated from livers of ANT-knockout mice have raised doubt on the current model of PTP [49] (see however [50]).

4.2. Classical vs. non-classical PT

Classical PT can be referred to as a process leading to CsA sensitive, ADP inhibitable matrix swelling. However, a CsA insensitive process has been observed under various conditions.
overcome by increasing Ca\(^{2+}\) loading into the matrix \([52,53]\). Relevant to the FFA effect considered here is the observation that FFA accumulation, as caused by mitochondrial phospholipase A\(_2\), may be a factor limiting the inhibitory effect of CsA on PTP \([54]\).

Other characteristics of the PTP appear to be more specific. Among these, the requirement for Ca\(^{2+}\) to induce PT appears absolute, while other divalent cations, such as Sr\(^{2+}\) or Mg\(^{2+}\), are inhibitory \([44]\). A characteristic feature is also represented by the membrane potential dependence of the PT. Titration experiments in intact mitochondria have shown that the PTP opening probability increases with increased depolarisation \([38]\). This aspect is of particular interest when the effect of FFA has to be considered (see above).

A non-classical permeability transition (NCPT) was reported by Sultan and Sokolove to occur on addition of palmitic acid (PA) or stearic acid (SA) to Ca\(^{2+}\)-loaded liver mitochondria \([55]\). Higher concentrations of both Ca\(^{2+}\) and PA than those generally used to induce the classical PT were required. Characteristics of the NCPT include the insensitivity to CsA and the interchangeability of Ca\(^{2+}\) with other divalent cations to stimulate PA-induced swelling. A model, distinct from the classical PT, was then proposed by these authors based on the formation of Ca\(^{2+}\)/PA pore complexes in the lipid bilayer. Importantly, agents known to inhibit the classical PT, ANT and the uncoupling protein (UCP) did not affect the PA induced NCPT \([55]\). NCPT is induced mainly by long chain saturated fatty acids, since the unsaturated ones, arachidonic acid in particular, failed to elicit the process under analogous conditions \([56]\). Consistently, it has been reported that (i) saturated FFA (C\(_{16}\)–C\(_{22}\)) bind Ca\(^{2+}\) by an affinity two orders of magnitude higher than other FFA \([57]\); (ii) PA or SA, but not unsaturated FFA, increases the conductance of the black-lipid membrane \([57]\). Moreover, it has been shown that PA or SA/Ca\(^{2+}\) complex caused the liposome-entrapped sulphorodamine B to be readily released from large unilamellar vesicles (LUV), whereas linolenic acid, when used instead of PA or SA, was ineffective \([58]\).

Energized heart mitochondria undergo classical PT upon addition of high (100–150 \(\mu\)M) Ca\(^{2+}\) concentrations \([43,52]\). The observed swelling is CsA and sanglifehrin A-sensitive and inhibited by ADP, with ATP exhibiting a smaller effect. Under the same experimental conditions, the addition of micromolar concentrations of arachidonic acid to 15–30 \(\mu\)M Ca\(^{2+}\)-loaded mitochondria respiring with succinate caused matrix swelling, which was largely CsA-insensitive and also supported by Sr\(^{2+}\) when used instead of Ca\(^{2+}\). BSA added after AA restored almost completely the membrane potential, inducing also a partial shrinking \([43]\). Unlike the classical PT, the AA-promoted CsA insensitive inner membrane permeability increase in heart mitochondria appears to be membrane-potential independent. In fact, it was shown that the addition of CCCP, at concentrations completely discharging the membrane potential, did not cause per se significant matrix swelling, induced by the subsequent addition of AA. Furthermore, AA and PA, both used at concentrations fully collapsing \(\Delta \Psi\), had very different effects on membrane permeability, with the unsaturated AA being much more effective than saturated PA in inducing swelling \([43]\). The conclusions that can be drawn from these experiments are 2-fold: (i) the AA-inducing permeability increase process is membrane potential-independent and (ii) based on the effect of unsaturated vs. saturated acids, it appears definitely different from the NCPT described above. The latter contention is further strengthened by the finding that the AA-induced swelling in CsA treated heart mitochondria is affected by ANT specific ligands. ATP was more effective than ADP in inhibiting, and atractyloside, at a concentration per se ineffective, stimulated AA-induced swelling (Table 1). Other nucleotides including GTP and GDP were almost ineffective.

These results led us to suggest that long chain unsaturated FFA may cause a Ca\(^{2+}\) mediated conversion of ANT into an ATP sensitive channel \([43]\). ANT can, in fact, become a pore at high matrix Ca\(^{2+}\) concentration \([59]\) and when the ATP/ADP binding sites are unoccupied \([44]\). Furthermore, an ATP inhibitable channel activity has been recorded in planar lipid membrane-reconstituted ANT \([47]\).

### 4.3. Distinct characteristics of matrix swelling in isolated heart and liver mitochondria

The extent of matrix swelling, as revealed by the absorbance decrease measured at 540 nm, is in heart mitochondria undergoing permeabilisation lower compared to that of liver mitochondria. This difference is likely to reflect the different number of contact sites, larger in heart mitochondria due to the presence of creatine kinase. Recent experiments using liver mitochondria isolated from transgenic mice expressing, unlike control animals, creatine kinase activity in this organ, have in fact shown that the enzyme was associated with the mitochondrial membranes, with the number of contact sites being increased 3-fold with respect to control mitochondria \([60,61]\).

Another interesting aspect, which appears relevant for understanding the tissue-specific structure/function of the putative PTP, is the different effect of FFA on membrane permeability increase in heart and liver mitochondria. Under exactly the same conditions as those employed in the experiments with heart mitochondria, succinate-energized liver mitochondria underwent classical CsA-sensitive PT upon addition of arachidonic or palmitic acid. Similarly, swelling was also induced by membrane depolarisation by CCCP \([43]\). The characteristics of AA-induced matrix swelling in mitochondria isolated from rat heart and liver are summarized in Table 2.

We have hypothesized that, with respect to the liver, higher concentrations of Ca\(^{2+}\) (as much as 1000–1500 nmol/mg protein) are required in heart mitochondria to assemble the various

<table>
<thead>
<tr>
<th>Matrix swelling</th>
<th>Extent ((\Delta A))</th>
<th>Rate ((\Delta A/\text{min}))</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.16 (+0.02)</td>
<td>0.101 (+0.05)</td>
</tr>
<tr>
<td>+Atr</td>
<td>0.21 (+0.027)</td>
<td>0.2 (+0.024)</td>
</tr>
<tr>
<td>+ATP</td>
<td>0.05 (+0.015)</td>
<td>0.022 (+0.0033)</td>
</tr>
</tbody>
</table>

ATP and atractyloside (Atr) were used at 0.2 and 0.1 mM, respectively. For experimental details and procedures see ref. \([43]\).
protein components, in particular Cyp. D, into PTP. If Cyp. D were not assembled, the system loses its sensitivity to CsA and sanglifehrin A, both of which bind to Cyp. D[62]. ANT, whose content in the heart is higher than in the liver, may still function as an ATP inhibitable, ΔΨ-independent channel, upon interaction with FFA. In the liver, where the candidate protein components of PTP are present, with the possible exception of Cyp. D[63], as tissue-specific isoforms, lower Ca2+ concentrations would suffice to assemble the classical CsA-sensitive PTP. The above hypothesis is supported by recent data showing that Cyp. D-deficient mitochondria were insensitive to CsA and exhibited a much higher Ca2+ retention capacity[64–66].

Relevant to this point are the results of experiments aimed at assessing the effect of AA on membrane permeability in heart mitochondria after treatment with diamide, which was shown to cross-link vicinal thiol groups on the ANT and to increase the sensitivity of PTP to Ca2+ and Cyp. D binding, probably, on the ANT Pro-61 in loop 1 at the matrix side[67]. We have indeed found that after treatment of heart mitochondria with diamide, the AA-induced matrix swelling exhibited the canonical features of classical PTP. It was in fact inhibited by CsA and specifically activated by Ca2+ (Sr2+ was ineffective). Under these conditions PT was also induced by membrane depolarisation (CCCP addition) (Fig. 2). The effect of diamide was prevented if DTT (0.5 mM) was present in the incubation mixture.

Thus, the already described Ca2+ sensitising effect caused by oxidative stress (diamide) [67] appears to result in heart mitochondria from the conversion of AA-induced CsA-insensitive ANT channel into the PT pore, most likely by favouring the assembly with ANT of other pore components, among which Cyp. D. The binding of the latter would account for the recovered sensitivity of the AA-induced swelling to CsA.

5. Conclusions

In this paper, we have outlined different, concentration-dependent effects and consequences ensuing upon interaction of FFA, arachidonic acid in particular, with mitochondria (Table 3).

A positive effect is exerted on the coupling efficiency of the bc1 complex by very low (submicromolar) concentrations of AA. This effect is additive with other conditions leading to decrease of transmembrane ΔpH[19]. Apparently, AA promote the activity of proton pump(s) by facilitating proton entry at the input mouth of the pump, in particular at high pH values of the negative matrix space.

Few micromolar FFA concentrations may give rise to a so-called mild uncoupling effect, which can prevent generation of ROS by mitochondria, at least those associated to membrane potential-supported reverse electron transfer from succinate to complex I.

When FFA accumulate, following a given stimulus, such as the activation of TNF-α receptor, there occur both a larger

![Graph](image-url)

**Fig. 2.** Effect of diamide on AA and CCCP-dependent swelling in heart mitochondria. Where indicated, diamide (250 μM) was added to the mitochondrial suspension 3 min before Ca2+. Swelling was induced by the addition of 10 μM AA (±2 μM CsA) or 0.25 μM CCCP to 30 μM Ca2+-loaded respiring rat heart mitochondria. For other experimental details and procedures see ref. [43].
uncoupling and inhibition of the respiratory chain enzymes. Generation of ROS under these conditions is now favoured. Such concentrations of FFA cause alteration of the inner membrane permeability with consequent matrix swelling and release of proapoptotic intermembrane proteins [43]. The characteristics of the AA-induced matrix swelling appear markedly different under the experimental conditions used, in mitochondria isolated from heart or liver (Table 1). While in the latter it presents the canonical features of classical PT, in heart mitochondria substantial differences were observed concerning, among others, the sensitivity to Ca\(^{2+}\). The dependence on the membrane potential and the inhibition by CsA. ANT, functioning as a non-selective ATP-sensitive channel, appears to be involved here. Upon oxidative stress this channel activity assumes, however, the characteristics of classical PTP.

The present observations may be relevant for the elucidation of the progress of mitochondrial dysfunction and cellular damage ensuing upon the heart ischemic and post-ischemic process. As the ischemic process starts, the initial AA accumulation may cause a CsA-insensitive increase of the inner membrane permeability of heart mitochondria (Fig. 3). This process is still reversible, depending on the effective concentration of AA (it is reversed by BSA), and is mainly controlled by the ATP concentration. In the subsequent reperfusion phase, when the AA concentration increases further [70] and ROS are produced largely, oxidative stress may cause Cyp. D to bind to ANT (Fig. 3). Under these conditions, the cell is likely committed to necrotic death. Relevant to this point is the report by Nakagawa et al., who showed that Cyp. D-deficient cells are resistant to necrotic death induced by ROS and Ca\(^{2+}\) overload, and that knock-out mice are highly resistant to ischemia/reperfusion-induced cardiac injury [64].

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