Fatty acid-promoted mitochondrial permeability transition by membrane depolarization and binding to the ADP/ATP carrier

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Abstract The mechanism by which non-esterified long-chain fatty acids (FFA) promote mitochondrial permeability transition (MPT) is not clear. We examined with energized rat liver mitochondria the role of two possible actions of FFA in MPT, (i) the reduction of the transmembrane potential ($\Delta\psi$) and (ii) the increase of the negative surface charge of the inner mitochondrial membrane [Broekemeier, K.M. and Pfeiffer, D.G., Biochemistry 43, (1995) 16440–16449]. It was found that the ability of FFA to stimulate large amplitude swelling is clearly related to their uncoupling activity. Moreover, compared with classical protonophores (FCCP) FFA increase the sensitivity of the pore opening process to $\Delta\psi$ changes. In addition, FFA interact like their thioester derivatives in a structure-dependent manner with the ADP/ATP carrier (measured as inhibition of [^{3}H]atractyloside binding to the AAC protein). It is suggested that not only the protonophoric action of FFA, but also a presumable stabilization of the 'cytosolic' conformation of AAC contribute to the FFA-promoted MPT.

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Key words: Fatty acid; Permeability transition; Liver mitochondrion; ADP/ATP carrier

1. Introduction

Elevated tissue levels of non-esterified long-chain fatty acids (FFA) can disturb the cellular energy metabolism in several ways. When they are applied at low concentrations (10–20 μM) to mitochondria, the proton electrochemical gradient ($\Delta\mu_{H^+}$) of the inner membrane is partly discharged by a FFA-mediated net transfer of protons to the matrix side [1–3], an event which uncouples oxidative phosphorylation [4]. There is now strong evidence that the ADP/ATP carrier (AAC) is involved in the FFA-mediated net transfer of protons (see [5,6] for review, and [7,8]). According to Skulachev [5], AAC facilitates the transmembrane movement of the fatty acid anion from matrix to cytosolic side (fatty acid circuit hypothesis). Furthermore, it has been shown that FFA are capable of translocating cations across lipid bilayers and biological membranes, presumably as the fatty acid cation soaps [9–11]. In addition, FFA can promote an unspecific increase of the inner membrane permeability to normally non-permeable solutes of much higher molecular mass [12–14]. When mitochondria are exposed to an excess of Ca^{2+} in the presence of so-called inducing agents (e.g. inorganic phosphate or sulfhydryl group reagents) they can undergo a sudden mitochondrial permeability transition (MPT) by opening of megachannels in the inner membrane (see [15,16] for review). MPT is associated with the loss of $\Delta\mu_{H^+}$, the release of endogenous Ca^{2+} and mitochondrial swelling. A characteristic feature of MPT is its specific inhibition by the immunosuppressant cyclosporin A [17,18]. Bernardi and coworkers have provided convincing evidence that the membrane potential ($\Delta\psi$) is an important modulator of MPT [19,20]. MPT is initiated when $\Delta\psi$ is decreased below the so-called gating potential. Moreover, it has been suggested that the promotion or the inhibition of MPT by 'effector agents' is due to a shift of the gating potential to lower or higher values [20]. Interestingly, there are indications for an involvement of AAC in MPT (see [16] for review and [21–23]). However, the mechanism by which FFA promote MPT is not clear. One possible mechanism might be that FFA decrease $\Delta\psi$ below the gating potential by acting as protonophores. Alternatively, it was proposed that FFA-promoted MPT is due to an increase in the negative surface charge as a consequence of insertion of their anionic forms into the inner mitochondrial membrane [14]. In addition, there is also the possibility that FFA could stabilize the 'cytosolic' conformation of the AAC protein, an event which is known to facilitate MPT (see for review [16]). In the present paper we have sought to define in incubation experiments with isolated rat liver mitochondria the action of FFA in MPT by testing these putative mechanisms. In conclusion, we propose that FFA promote MPT by decreasing the $\Delta\psi$ as consequence of their protonophoric properties.

2. Materials and methods

2.1. Mitochondria

Liver mitochondria with respiratory control ratios routinely higher than 5 were prepared from adult female Wistar rats (mean weight 150–180 g) according to our standard protocol [2]. The protein content in the mitochondrial stock suspension was determined by a modified biuret method.

2.2. Measurements

The permeability transition of energized rat liver mitochondria was followed by the absorbance change at 540 nm resulting from permeation of sucrose plus mannitol in the mitochondrial matrix. Swelling was recorded at room temperature with a Cary UV-Visible Spectrophotometer. In short, 1 mg of mitochondrial protein was added to 1 ml of swelling medium, consisting of 210 mM mannitol, 70 mM sucrose, 10 mM Tris, 5 mM glutamate, 5 mM malate, pH 7.4. To define the incubation conditions for the pore opening process that were most sensitive to protonophores, mitochondria were incubated in preliminary experiments with swelling medium supplemented with 5 mM phosphate, various Ca^{2+} concentrations and 0.1 μM FCCP. A mitochondrial Ca^{2+} load of 70 nmol/mg protein was applied throughout. The effect of added FFA on $\Delta\psi$ was determined in parallel incubations, but in phosphate-free swelling medium to prevent MPT.

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Abbreviations: FFA, non-esterified long-chain fatty acids; AAC, ADP/ATP carrier; [^{3}H]ATR, atractyloside; Phyt, phytanic acid; FCCP, *p*-trifluoromethoxycarbonyl-cyanide phenylhydrazone; $MPT_{50\%}$, time of half-maximal swelling of mitochondrial fraction

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Dissipation of $\Delta\psi$ by FFA was estimated from the distribution of the highly membrane-permeable lipophilic [^{3}H]MTPP$^+$ between the matrix compartment and the surrounding medium. Briefly, mitochondria were separated from an aliquot of the incubation mixture (0.2 ml) by centrifugation of mitochondria through silicon oil (density 1.037). The radioactivity was counted in an aliquot of the supernatant and of the solubilized pellet in 2% sodium dodecyl sulfate. The measured radioactivity in the pellet was corrected for unspecific binding of [^{3}H]MTPP$^+$ to deenergized mitochondria. $\Delta\psi$ was calculated from triplicate countings adopting as matrix space volume 1 μl/mg protein.

The interaction of FFA with the AAC protein was estimated from the effect of FFA on [^{3}H]atractyloside binding to AAC as described in [24].

2.3. Chemicals

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), eicosanoic acid (C20:0), sodium dodecyl sulfate (SDS) and triphenylacetic acid, atractyloside, carboxyatractyloside, FCCP, ruthenium red, palmitoyl-CoA, cyclosporin A, glutamate and malate were from Sigma (St. Louis, MO, USA). [^{3}H]MTPP$^+$ and [^{3}H]ADP were obtained from NEN-DuPont (Boston, MA, USA).

3. Results

3.1. Mitochondrial permeability transition

To study the role of FFA in MPT, energized RLM were incubated with μM concentrations of various FFA (differing in structure and protonophoretic activity) or non-lytic concentrations of SDS. SDS, a surface active agent without protonophoretic activity, was used as a tool for changing the surface charge of the inner mitochondrial membrane. Swelling was triggered by the addition of phosphate. Fig. 1 shows that FFA differ in their ability to promote Ca^{2+}-dependent MPT as indicated by large amplitude swelling. Palmitic and phytanic acids rapidly promote MPT, whereas with eicosanoic acid (an unbranched isomer of phytanic acid) or triphenylacetic acid (not shown) the initiation of swelling is delayed. Under the same conditions SDS did not promote MPT. The FFA-promoted MPT is a function of the mitochondrial Ca^{2+} load (Fig. 2). When phytanic acid was applied in lower concentrations the traces of swelling were always sigmoidal, which is explained by the uptake of Ca^{2+} ions released from permeabilized mitochondria by mitochondria whose pores are still closed [19,25]. When this swelling-linked Ca^{2+} redistribution was omitted with the Ca^{2+} uniport inhibitor ruthenium red (RR) [12], higher concentrations of FFA were needed to adjust large amplitude swelling. Without and with RR-treated mitochondria promotion of swelling by FFA is blocked in the presence of 1 μM of the immunosuppressant cyclosporin A (not shown).

3.2. Protonophoric activity and swelling

Classical protonophores such as FCCP stimulate MPT by decreasing $\Delta\psi$ below the gating potential [19,20]. To examine the effect of a FFA-induced reduction of $\Delta\psi$ on MPT, the stimulation of swelling by FFA was measured with RR-treated mitochondria to omit the effect of Ca^{2+} redistribution. Mitochondria were incubated in the medium supplemented with phosphate (5 mM), Ca^{2+} (70 nmol/mg protein) and various FFA or SDS (30 nmol/mg protein). In parallel incubations (without Ca^{2+}) the depression of $\Delta\psi$ by these FFA was measured. The measured data for the absorption decrease were plotted vs. corresponding $\Delta\psi$ data (Fig. 3). From these experiments it follows clearly that the rate of swelling stimulated by fatty acids is related to the decrease in $\Delta\psi$.

Furthermore, mitochondria were treated in separate incubations with increasing concentrations of phytanic acid (2.5–12.5 nmol/mg protein) as in Fig. 2A, and the time for half-maximal swelling ($MPT_{50\%}$) was measured (Fig. 4). The same measurements were done with FCCP as MPT promoter. When the obtained $MPT_{50\%}$ data were plotted against corresponding $\Delta\psi$ data, it became evident that pore opening is gated by similar changes in $\Delta\psi$ for both types of protonophores. However, compared with FCCP, the range of $\Delta\psi$-gated pore opening was shifted to slightly higher $\Delta\psi$ data when phytanic acid and palmitic acid (not shown) were applied.

3.3. Interaction of FFA with AAC

Previously we reported that FFA can bind to the AAC protein like their CoA derivatives [8]. Therefore, it could be that FFA promote MPT additionally by stabilizing the 'cytosolic' conformation of the AAC, similar as known for carboxyatractyloside, atractyloside or the CoA derivatives of long-chain fatty acids [16]. To compare the interaction of various FFA with the AAC protein, mitochondria were first preincubated with FFA and then treated with [^{3}H]ATR. The residual binding of [^{3}H]ATR to mitochondria reflects the affinity of FFA to AAC. Fig. 5 shows that in comparison to palmitoyl-CoA, the affinity of applied FFA to the AAC protein is smaller. Palmitic and phytanic acids compete effectively with [^{3}H]ATR for binding to AAC, whereas lauric and eicosanoic acids were less effective.

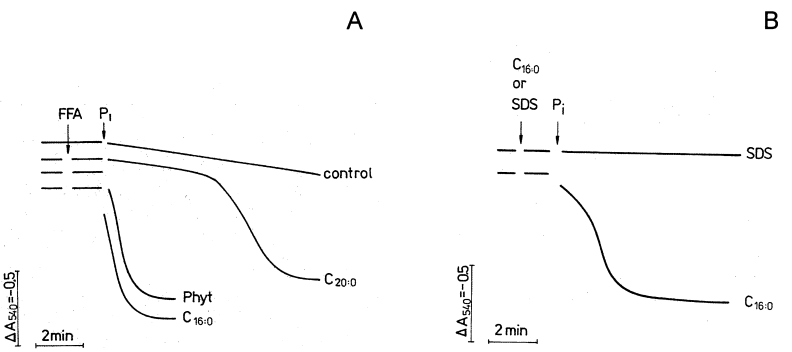


Fig. 1. Promotion of the permeability transition by fatty acids. RLM (1 mg protein) were loaded in 1 ml swelling medium with 70 nmol Ca^{2+}/mg protein. Additions were done as indicated: (A) 30 nmol FFA/mg protein or (B) 30 nmol SDS/mg protein. Swelling was triggered by 5 mM phosphate.

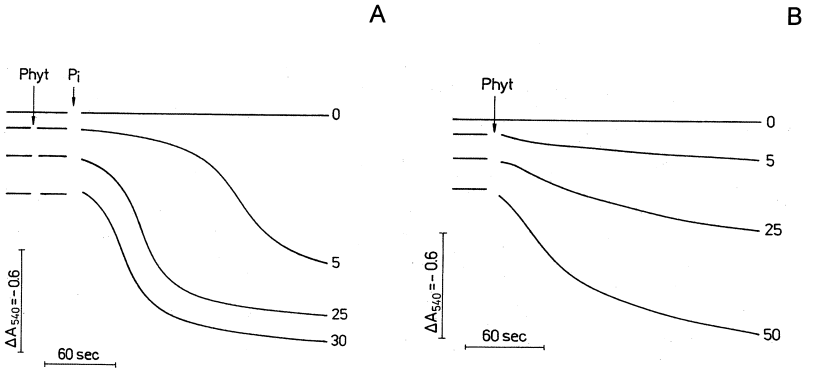


Fig. 2. Promotion of permeability transition by phytanic acid in the absence and presence of ruthenium red. A: RLM (1 mg protein) were first loaded with 70 nmol Ca^{2+}/mg protein, followed by addition of various concentrations of phytanic acid as indicated in nmol/mg protein at the traces of swelling curves. B: RLM (1 mg protein) were preincubated 1 min in the swelling medium supplemented with 5 mM phosphate plus 70 nmol Ca^{2+}/mg protein, followed after 1 min by addition of 1 μM ruthenium red. Ca^{2+} redistribution between mitochondria was omitted in the presence of ruthenium red. Swelling was initiated by addition of various concentrations of phytanic acid.

4. Discussion

We show in this study that the ability of FFA to promote MPT is a function of their structure and the applied concentration (Figs. 1 and 2). Palmitic and phytanic acids were found to be 'good' MPT promoters, whereas eicosanoic acid and triphenylacetic acid are less effective. The efficacy of these FFA to promote MPT is in line with their ability to stimulate uncoupled mitochondrial respiration (results not shown). From the different types of possible interactions between FFA and components of the mitochondrial inner membrane (reduction of Δψ; increase of negative surface charge; stabilization of the 'cytosolic' conformation of AAC) we show that the promotion of MPT by FFA is related to their protonophoric property, which is supported by the correlation between the rate of swelling and Δψ reduction (Fig. 3). FFA facilitate the transmembranal net proton transfer probably by flip-flop movement, which is partly assisted by the AAC protein and, in addition, membranal proteins such as the dicarboxylate carrier and the aspartate/glutamate carrier [26,27]. From bilayer experiments it is known that the flip-flop movement of FFA with unbranched chain length (C12:0 to C18:0) is more rapid (half time: 0.08 s) than that of eicosanoic acid (half time: 16 s [28]). Therefore, the different abilities of FFA to exert flip-flop movement could explain that very long-chain fatty acids (eicosanoic acid) are much less active in uncoupling (Fig. 3) and in promoting MPT than palmitic acid (Fig. 1).

Furthermore, an increase of the negative surface charge by FFA resulting from insertion of their anionic forms into the inner mitochondrial membrane seems to be of minor importance for their ability to promote MPT. Neither SDS nor eicosanoic acid or triphenylacetic acid is as potent as palmitic or phytanic acid to promote MPT (Fig. 1) or to stimulate swelling (Fig. 3). In addition, the promotion of MPT by FFA responded very sensitively to the mitochondrial Ca^{2+} load (Fig. 2). Moreover, keeping in mind that FFA are capable of translocating cations across membranes, there is reason to discuss that they might contribute to pore opening by mediating Ca^{2+} uptake from the medium to the mitochondrial matrix. However, we suggest that such an action of FFA under our conditions is not very active. Were such an action

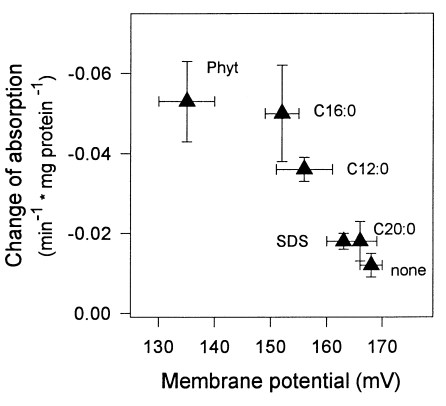


Fig. 3. Effect of the FFA-induced decrease of the membrane potential on the swelling rate. From experiments shown in Fig. 2B the rate of initial absorption decrease was calculated by means of the photometer software. In separate incubations, the effect of various fatty acids and that of SDS (30 nmol/mg protein) on the membrane potential was measured in the absence of phosphate. The data points represent the means obtained from three preparations.

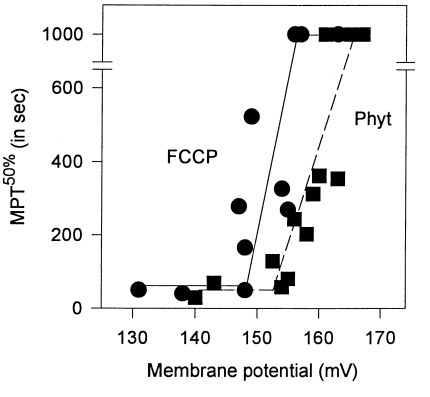


Fig. 4. Time-course of the permeability transition as a function of membrane depolarization. RLM (1 mg protein) were treated with various concentrations of phytanic acid or FCCP as in Fig. 2A. Swelling was triggered by the addition of 5 mM phosphate. The reduction of Δψ by phytanic acid or FCCP was estimated with the $[^3H]MTPP^+$ cation as Δψ probe, but under conditions where mitochondria did not undergo MPT (without Pi). The data were obtained from three mitochondrial preparations.

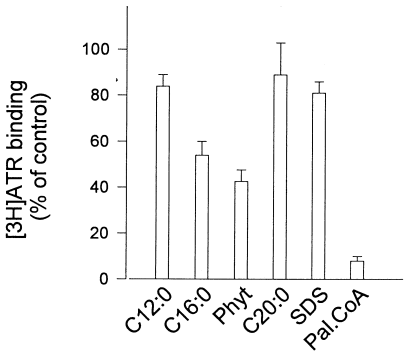


Fig. 5. Effect of fatty acids on the binding of [^{3}H]atractyloside to mitochondria. RLM (1 mg protein) were preincubated for 1 min with 100 nmol/mg protein of various fatty acids, palmitoyl-CoA or SDS. After that, RLM were equilibrated with 1 nmol [^{3}H]ATR (specific activity 220 dpm/pmol). Non-specific binding of [^{3}H]ATR to mitochondria was determined by incubation of the mitochondrial samples with 100 μM unlabeled atractyloside prior to addition of [^{3}H]ATR. The data are the means ± S.D. obtained from 4–7 mitochondrial preparations.

to be active, then also in incubations with RR-treated mitochondria low concentrations of FFA should promote MPT.

Finally, the sensitivity of MPT to the ligand-induced change in the AAC conformation is well-established. Ligands of AAC which stabilize its 'cytosolic' conformation (carboxyatractyloside, atractyloside, acyl-CoA derivatives) or those stabilizing its 'matrix' conformation (ADP or bongkrekig acid) facilitate or inhibit MPT [16]. Our study shows that fatty acids increase the sensitivity of the pore opening to $\Delta\psi$ changes in comparison to classical protonophores (FCCP) (Fig. 4). In addition, we show that FFA interact like their thioester derivatives [29] in a structure-dependent manner with the AAC protein (Fig. 5). Both these findings suggest that not only the protonophoric action of FFA, but also a presumable stabilization of the 'cytosolic' conformation of AAC might contribute to the FFA-promoted MPT.

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