Fatty acids decrease mitochondrial generation of reactive oxygen species at the reverse electron transport but increase it at the forward transport

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Abstract

Long-chain nonesterified (“free”) fatty acids (FFA) can affect the mitochondrial generation of reactive oxygen species (ROS) in two ways: (i) by depolarisation of the inner membrane due to the uncoupling effect and (ii) by partly blocking the respiratory chain. In the present work this dual effect was investigated in rat heart and liver mitochondria under conditions of forward and reverse electron transport. Under conditions of the forward electron transport, i.e. with pyruvate plus malate and with succinate (plus rotenone) as respiratory substrates, polyunsaturated fatty acid, arachidonic, and branched-chain saturated fatty acid, phytanic, increased ROS production in parallel with a partial inhibition of the electron transport in the respiratory chain, most likely at the level of complexes I and III. A linear correlation between stimulation of ROS production and inhibition of complex III was found for rat heart mitochondria. This effect on ROS production was further increased in glutathione-depleted mitochondria. Under conditions of the reverse electron transport, i.e. with succinate (without rotenone), unsaturated fatty acids, arachidonic and oleic, straight-chain saturated palmitic acid and branched-chain saturated phytanic acid strongly inhibited ROS production. This inhibition was partly abolished by the blocker of ATP/ADP transfer, carboxyatractyloside, thus indicating that this effect was related to uncoupling (protonophoric) action of fatty acids. It is concluded that in isolated rat heart and liver mitochondria functioning in the forward electron transport mode, unsaturated fatty acids and phytanic acid increase ROS generation by partly inhibiting the electron transport and, most likely, by changing membrane fluidity. Only under conditions of reverse electron transport, fatty acids decrease ROS generation due to their uncoupling action.

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1. Introduction

Generation of “reactive oxygen species” (ROS) by mitochondria was first described by Chance and co-workers [1–3]. Superoxide (O2−) is formed as side-product of the respiratory chain by one-electron transfer reactions to molecular oxygen within complex I (NADH-ubiquinone oxidoreductase) and complex III (ubiquinol-cytochrome c oxidoreductase) [4,5]. In contrast to complex III, where O2− generation is attributed to ubisemiquinone as electron donor [3,6,7], the site of complex I-associated O2− generation is still under discussion. Fe–S clusters [8], the flavine mononucleotide [9,10] and the region around ubisemiquinone-binding sites [11] have been discussed as electron donor sites. Moreover, O2− is released from complex I to the matrix side of the inner membrane, whereas complex III releases O2− to both sides of the inner membrane [12–14]. O2− is rapidly converted to H2O2 by the Mn-containing superoxide dismutase (inside mitochondria) or the Cu,Zn-containing superoxide dismutase (intermembrane space and cytosol) [5]. H2O2 is decomposed by catalase or reduced to H2O by glutathione peroxidase.

Abbreviations: AA, antimycin A; Ara, arachidonic acid; CDNB, 1-chloro-2,4-dinitrobenzene; CAT, carboxyatractyloside; Cyt c, cytochrome c; FCCP, carbonyl cyanide 4-trifluoro-methoxyphenylhydrazone; FET, forward electron transport; FFA, free fatty acids; RHM, rat heart mitochondria; RLM, rat liver mitochondria; Rot, rotenone; ROS, reactive oxygen species; Lin, linoleic acid; Mal, malate; Myr, myristic acid; Ole, oleic acid; Pal, palmitic acid; Phyt, phytanic acid; Pyr, pyruvate; RET, reverse electron transport; Succ, succinate; Δψm, mitochondrial membrane potential

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It has been found that the transition of succinate-oxidizing mitochondria from state 4 (resting state) to state 3 (active state) decreases respiratory chain-associated ROS generation [2]. Similarly, the chemical protonophore FCCP or free long-chain fatty acids (FFA) decrease ROS generation by succinate-oxidizing rat heart mitochondria [15,16]. These observations indicate that even a slight depolarisation of the inner mitochondrial membrane abolishes mitochondrial ROS generation (mild uncoupling concept, [7]). The mild uncoupling concept has stimulated a lot of experimental work to stress the role of the mitochondrial membrane potential (Δψm), and also that of the uncoupling proteins (for review see [17]), in mitochondrial ROS generation. From these studies, mostly done with isolated mitochondria from rat brain and heart, the following conclusions can be drawn: (i) mitochondria oxidizing NAD-linked substrates (e.g. pyruvate plus malate) generate a low level of ROS by complex I and, in addition, this level decreases only moderately by depolarisation with FCCP [18–20]. (ii) Mitochondria oxidizing NAD-linked substrates respond to rotenone (complex I inhibitor) or antimycin A (complex III inhibitor) with a dramatic increase in ROS generation, although both inhibitors collapse Δψm. (iii) In contrast, when complex I is supplied with electrons deriving from the reversed electron transport (RET), mitochondrial ROS generation decreases severely after addition of rotenone or FCCP [15,18]. (iv) ROS generation by mitochondria respiring with succinate plus rotenone is poor, but increases dramatically after addition of antimycin A. (v) Depletion of cytochrome c from mitochondria increases ROS generation [21].

Tissues contain small amounts of FFA, which are mostly bound to proteins and membranes [22]. The concentration of total nonesterified fatty acids in human plasma is about 0.5 mM and can be tripled under fasting [23] or doubled under physical exercise [24]. FFA also accumulate in tissues during ischemia and in peroxisomal diseases [25,26]. There is evidence that FFA are involved in ischemia arrhythmias, reperfusion injury, cardiac dysfunction in diabetes and impaired insulin secretion as well as insulin action [25,27–29]. There is reason to assume that FFA are present in tissues at a similar concentration range as in blood. However, their actual distribution between various cellular structures is not known. Nevertheless, it seems that the concentrations of FFA applied in the present study (80 μM maximum), similar to those used by other cited authors, are within the physiological or pathological conditions. As protonophoric uncouplers and inhibitors of the respiratory chain [30–33], FFA are able to modulate mitochondrial ROS generation by uncoupling and interfering with the electron transport. In addition, FFA can also impair the electron transport as consequence of the release of cytochrome c from the external side of the inner membrane [21,34].

To date, the available data on the effect of FFA on mitochondrial ROS generation are controversial. As mentioned above [16], FFA abolish ROS generation by rat heart mitochondria under condition of RET (succinate as substrate), whereas FCCP-uncoupled rat heart mitochondria respond to high concentrations of FFA with increased ROS generation [31]. In addition, stimulation of ROS generation by FFA was also reported from a study using rat brain and heart mitochondria respiring with NAD-linked substrates in rest [33]. Consequently, the mechanism underlying the modulation of mitochondrial ROS by FFA remains unclear. To get more insight into these relationships, in the present investigation we treated rat heart and liver mitochondria with low, micromolar, concentrations of various fatty acids, including saturated, unsaturated and branched-chain FFA. The results demonstrated that the protonophoric action of FFA does not significantly affect ROS generation by mitochondria at the forward electron transport (FET). On the opposite, some FFA (especially Phyt, Lin and Ara) remarkably stimulate ROS, whereas Pal increases ROS generation only at a relative high concentration. Finally, ROS generation associated with complex III is reciprocally correlated with the inactivation of its enzymatic activity by FFA.

2. Materials and methods

2.1. Preparation of mitochondria

Heart mitochondria were isolated by differential centrifugation from tissue homogenates obtained from adult female Wistar rats (average weight 150–180 g) essentially as described in [35]. Before tissue homogenisation, pieces of heart tissue were treated with trypsin. The mitochondrial pellet was resuspended in 0.25 M sucrose at a concentration of 20–12 mg×ml−1. Liver mitochondria were prepared by differential centrifugation of tissue homogenates as described in [36]. The mitochondrial pellet was resuspended in 0.25 M sucrose at a concentration of 25–35 mg×ml−1. Protein contents in the stock suspensions were determined by biuret method using bovine serum albumin as standard. Functional integrity of mitochondrial preparations was estimated by the respiratory control ratio, which was routinely higher than 10 using pyruvate plus malate (RHM) and 8 using glutamate plus malate (RLM) as substrates.

The standard incubation medium was composed of 110 mM mannitol, 60 mM KCl, 60 mM Tris–HCl, 10 mM KH2PO4, 0.5 mM EGTA (pH 7.4). The medium was supplemented with 5 mM Pyr plus 5 mM Mal or with 10 mM Succ as respiratory substrates. Stock solutions of fatty acids and respiratory chain inhibitors were made in ethanol.

2.2. Oxygen uptake

Oxygen uptake by mitochondria was measured using an oxygraph (Ororobos Oxygraph®, Bioenergetics and Biomedical Instruments, Innsbruck, Austria).

2.3. ROS generation

ROS generation was estimated as the release of H2O2 from mitochondria. For this purpose oxidation of Amplex Red (non-fluorescent) to resorufin (fluorescent) was followed fluorimetrically [37]. Briefly, mitochondria (0.2 mg mitochondrial protein × ml−1) were incubated in the standard incubation medium supplemented with 5 μM Amplex Red plus horseradish peroxidase (2 U × ml−1) to detect H2O2 and Cu,Zn-superoxide dismutase (2 U × ml−1) for quantitative conversion of released O2 to H2O2. Resorufin fluorescence was monitored by Perkin-Elmer Luminescence spectrometer LS 50B (excitation at 560 nm, emission at 590 nm). The increase in resorufin fluorescence was calibrated with H2O2. Addition of ascorbic acid or phytic acids at the concentrations used in this work had no effect on the calibration curve.

2.4. Complex III activity

Complex III activity was measured essentially as described by Trumpower and Edwards [38]. Before measuring enzymatic activity, mitochondria were...
permeabilized by freezing/thawing cycles (3 times) and then exposed to FFA. Absorption changes were measured in the standard incubation medium using a Cary 3E UV-Visible Spectrophotometer.

Ubiquinol–cytochrome c oxidoreductase (complex III) was assayed by monitoring the initial rate of reduction of oxidized cytochrome c by decylubiquinol at 550 nm ($\varepsilon = 19.2 \text{ cm}^2 \cdot \mu \text{mol}^{-1}$). As described above, all components (including 10 µg of the mitochondrial sample) except decylubiquinol (50 µM) were added into both the sample and the reference cells. Decylubiquinol was reduced by adding a small crystal of NaBH$_4$ to a solution of commercial decylubiquinone in ethanol. When the reaction mixture became colourless, the liquid was transferred to another tube (omitting the crystal), acidified by addition of 0.1 volume of 1 M HCl and stored at −20 °C [39].

2.5. Chemicals

Fatty acids (except phytanic acid), FCCP, rotenone, antimycin A, l-chloro-2,4-dinitrobenzene (CDNB), Cu,Zn-superoxide dismutase, horseradish peroxidase (Type VI-A), decylubiquinone, cytochrome c and carboxyatractyloside were from Sigma-Aldrich Chemie GmbH (Sternheim, Germany). Phytanic acid was supplied by ULTRA Scientific (North Kingstown, RI) and Amplex Red by Invitrogen (Eugene, Oregon). Trypsin was from Serva (Heidelberg, Germany).

2.6. Data analysis

Statistical analysis was performed using Prism 3.0 software (GraphPad Software, San Diego, CA). Significance was examined by the unpaired $t$ test. $p < 0.05$ was considered statistically significant with respect to control incubation.

3. Results

3.1. FFA stimulate ROS generation supported by the forward electron transport (FET)

FFA can modulate ROS generation in mitochondria in state 4 either by uncoupling or by interference with the respiratory chain. The effect of FFA on ROS generation accompanying FET was studied in rat heart and liver mitochondria supplied with Succ (plus Rot) or Pyr plus Mal and treated with 20, 40 and 80 µM of Pal, Phyt or Ara (corresponding to 100, 200 and 400 nmol FFA per mg protein). Fig. 1 shows that ROS generation increased with increasing concentrations of Phyt or Ara. FFA-linked ROS generation was higher with Succ (plus Rot) than with Pyr plus Mal (compare panels A and B or C and D). In RHM, Phyt was the most potent inducer of ROS (panels A and B), whereas in RLM Phyt and Ara produced a similar increase in ROS generation (panels C and D). In contrast, Pal only slightly stimulated ROS generation.

![Fig. 1. Stimulation of ROS generation by increasing concentrations of Pal, Ara and Phyt. Mitochondria from heart or liver were incubated with Pyr plus Mal or Succ plus Rot. FFA were added to final concentrations of 20, 40 and 80 µM, corresponding to 100, 200 and 400 nmol x mg protein$^{-1}$, ROS production in the absence of Rot, i.e. under conditions of RET, in the absence of FFA is also shown for comparison (full squares). Data shown are means±SD obtained from 4 to 6 mitochondrial preparations.](image-url)
3.2. FFA abolish ROS generation supported by the reverse electron transport (RET)

The effect of FFA on ROS production accompanying RET was investigated in mitochondria oxidizing Succ in the absence of Rot (Fig. 2). Firstly, it has to be noted that omission of Rot remarkably increased ROS production from about 30 to about 250 pmol × min⁻¹ × mg protein⁻¹ for RHM (Fig. 1, panel B) and from about 25 to about 60 pmol × min⁻¹ × mg protein⁻¹ for RLM (Fig. 1, panel D). Pal, Phyt, Ole and Ara (20 μM, corresponding to 100 nmol FFA per mg protein) strongly decreased ROS generation, similarly as the chemical uncoupler FCCP (Fig. 2, panel A). Even such a low concentration of FFA as 1 μM (corresponding to 5 nmol × mg protein⁻¹) diminished ROS production by 50% (Fig. 2, panel B). This decrease of RET-linked ROS production by FFA was most likely due to their protonophoric activity, which prevents the RET from Succ to complex I by decreasing Δψm. Protonophoric activity of FFA is explained by a cyclic movement of protonated and deprotonated forms of fatty acids across the inner mitochondrial membrane, mostly supported by the adenine nucleotide antiporter [40,41] that can be inhibited by carboxyatractyloside (CAT). High recoupling by CAT has been observed in FFA-uncoupled heart mitochondria [42]. Therefore, the effect of recoupling on Pal-suppressed ROS production was examined. Indeed, recoupling by CAT (indicated as a decrease of respiration) was paralleled by reactivation of ROS production (Fig. 2, panel C).

3.3. Fatty acid uncoupling does not change fatty acid-induced ROS generation

In contrast, when RET was blocked by Rot, electrons were transported from Succ exclusively to complex III (FET). At this situation, the basal ROS production was lower and was stimulated by FFA (Fig. 1). It could be hypothesised that this stimulation was the resultant of an inhibitory effect of FFA on the electron transport through the respiratory chain. Addition of Phyt to RHM fuelled with Succ (plus Rot) in state 4 stimulated the respiration in a concentration-dependent manner that was partly reversed by CAT (Fig. 3, panels A and C). It can also be seen that immediately after addition of the highest applied concentration of Phyt (Fig. 3, panel A, trace c; 100 nmol Phyt per mg protein), the respiration was first stimulated, but slowed down thereafter. Stimulation of respiration reflects the uncoupling activity of Phyt, which induced the depolarisation of the inner membrane. However, in contrast to the recoupling effect of CAT on respiration, Phyt-stimulated ROS generation was not changed by CAT (Fig. 3, panels B and C). These rates of respiration and corresponding ROS generation are summarized in panel C. It can be seen that CAT partly reversed FFA-associated increase of respiration, but was without effect on ROS generation. These findings clearly indicate that stimulation of ROS production at FET by such FFA as Myr, Phyt or Lin was not affected by their protonophoric activities.

3.4. Fatty acids stimulate ROS generation in glutathione-depleted mitochondria

Mitochondrial ROS generation is buffered by the matrix content of reduced glutathione. Thus, depletion of glutathione enhances ROS generation [43,44]. With this in mind, we examined whether depletion of glutathione could affect ROS generation by FFA.
production by glutathione-depleted RHM (Fig. 4, panel B). Such stimulation of ROS generation by FFA in glutathione-depleted mitochondria clearly indicates that FFA-associated ROS generation in untreated mitochondria was most likely due to an interference with the electron transport rather than with the amount or the rate of generation of reduced glutathione within the mitochondrial compartment (see Discussion).

3.5. Complex III-associated ROS generation correlates with inactivation of its enzymatic activity

As demonstrated above, the stimulation of ROS generation by FFA was more steeply dependent on FFA concentration in case of Succ oxidation than with NAD-linked substrates (Fig. 1). Therefore, the enzymatic activity of complex III (expressed as the rate of decylubiquinol oxidation by oxidised Cyt c) was measured at increasing concentrations of Pal, Phyt and Ara. It was found that in RHM the enzymatic activity of complex III became strongly decreased with increasing concentrations of
Phyt or Ara, whereas Pal was less inhibitory to complex III (Fig. 5, panel A). In contrast, Pal and Ara were similarly inhibitory to complex III in RLM, but both these FFA were weaker inhibitors than Phyt (Fig. 5, panel B). Rates of ROS generation and enzymatic activities were plotted against each other in Fig. 6. These plots show that for Phyt and Ara there was a reverse linear correlation between complex III activity and ROS generation in heart mitochondria (Fig. 6, panel A) over the whole range of complex III activity. In contrast, in RLM ROS generation was not affected by partial inhibition of the respiratory activity by up to about 30% for Ara and by up to 70% by Phyt (Fig. 6, panel B). Also surprisingly and in contrast to RHM, total inhibition of complex III by antimycin A in RLM was accompanied by a negligible increase in ROS production only.

3.6. Fatty acids enhance ROS production at blocked complex I

It is known that incorporation of FFA in the inner mitochondrial membrane increases the membrane fluidity [46,47], which can change membrane-associated energy coupling processes [48]. Therefore, it can be hypothesised that FFA facilitate the access of O₂ to electron-donating sites within the respiratory chain and, in addition, the release of O₂⁻. To examine such possibility, the effect of various FFA was studied on RHM suspended in the standard medium containing Pyr plus Mal additionally supplemented with Rot, the situation where the electron transport via complex I was blocked. It was found that under such condition addition of Rot considerably enhanced ROS generation and that this increase was further enhanced by FFA (Fig. 7). Phyt and Lin strongly stimulated ROS production, Ara was somewhat less effective and Pal exerted only slight effect if any. Furthermore, stimulation of ROS production by these FFA was found also when RHM were incubated with 2-oxoglutarate plus Rot or succinate plus Rot plus antimycin A (not shown). In conclusion, these observations indicate that FFA can enhance O₂⁻ production under conditions where the electron transport by complex I or complex III is blocked. Similar results were also obtained with brain mitochondria (not shown).

4. Discussion

It has been reported that ROS production by mitochondria under resting state conditions (state 4) is more or less decreased by depolarisation of the inner membrane [15,18–20]. In isolated
mitochondria, this decline depends on the respiratory substrate used. Thus, mitochondria oxidising Succ generate high amounts of ROS due to RET. This generation is abolished even by a slight decrease of Δψm [2,15]. The reason why such thermodynamically unfavourable backward transport of electrons from complex III to NAD\(^+\) generates more O\(_2^-\) than their forward transport from NADH through complex I to complex III is not fully understood. Transition from state 4 to state 3, accompanied by some lowering of Δψm, is associated with a considerable decrease of ROS production in Succ-oxidising mitochondria which can be returned to its state 4 level by addition of CAT (blocking ADP/ATP exchange) or oligomycin (blocking ADP phosphorylation) [49]. As expected, ROS production by mitochondria functioning in the RET mode is strongly decreased by Rot, since the O\(_2^-\) production in complex I (most likely related with its FMN moiety) is upstream of the site where Rot blocks the electron transport (for review see [50]).

The physiological role of RET is strongly debatable and so are its possible functions in in vivo ROS generation and the resulting tissue damage, e.g. in ischemia/reperfusion or neurodegenerative diseases (discussed in [51]).

In contrast, ROS generation is relatively low in mitochondria energized with NAD\(^+\)-linked substrates (e.g., Pyr plus Mal), despite that mitochondria are highly energized and matrix NAD\(^+\) is mostly reduced. The same is true for mitochondria oxidising Succ in the presence of Rot, i.e., under conditions supporting FET. Under these situations, ROS production is also much less sensitive to agents dissipating Δψm [18,19,20,52] but is strongly increased by blockers of the respiratory chain, like Rot [21] and antimycin A [6], although the latter agents collapse Δψm.

Since FFA are not only physiological protonophores but also inhibitors of the ADP/ATP exchange and of the electron transport (for review see [22]), they can be expected to modulate the mitochondrial ROS production in different ways. In fact, it was observed in the present work that unsaturated long-chain fatty acids, Lin and Ara, and the saturated branched-chain fatty acid Phyt stimulated ROS production, whereas saturated fatty acids Myr and Pal were less active or without effect (Figs. 1, 3 and 4). This observation was remarkable, as all these FFA acted as week uncouplers (Figs. 2 and 3) by increasing resting state respiration in a CAT-sensitive way [40,41]. Moreover, unsaturated fatty acids and Phyt appeared to decrease the rate of uncoupled respiration with both NAD-linked substrates and Succ (plus Rot) [30,31,33]. Interestingly, at higher concentrations they were also inhibitory in state 4 respiration [30,31,33]. This finding confirms the long-known dual effect of FFA on mitochondrial respiration (also reviewed in [22]): protonophoric action on the inner mitochondrial membrane and inhibitory action on the electron transfer chain.

Having this in mind, we may explain stimulation of mitochondrial ROS production by unsaturated fatty acids and Phyt as being due to the inhibition of electron transport, the effect similar to that exerted by antimycin A or rotenone. Thus, by inhibition of the electron transport across complexes I and III of the respiratory chain, FFA promote the one-electron reducing mechanism of molecular oxygen. A similar increase of ROS production by Pal and Ara was found in FCCP-uncoupled RHM by Cocco et al. [31]. Interference of FFA with the mitochondrial electron transport is well documented [30–33], although its molecular mechanism is not well understood. Interestingly, the respiratory chain as a whole and its complexes I and III have also been shown to be inhibited by fatty acid derivatives ceramide [53] and N-arachidonoyl ethanolamine [54]. Both these compounds also increased mitochondrial ROS production under specific conditions [53,54]. Here we have shown that complex III becomes inactivated by Pal, Phyt and Ara in permeabilized mitochondria in a concentration-dependent manner (Fig. 5).

Despite a direct inhibitory action of FFA at certain sites of the electron transport chain, FFA could induce mitochondrial deploleletion of Cyt c [27,34,55]. In addition, incorporation FFA into the mitochondrial membrane was found to increase its fluidity [46–48], an interaction that may likely affect functioning of the respiratory chain and, presumably, also the production of O\(_2^-\). Thus, treatment of beef heart submitochondrial particles with erucic acid (cis-13-docosenoic acid) increased O\(_2^-\) production and membrane fluidity [47]. In addition, we have shown that FFA enhance the release of O\(_2^-\) from complex I in Rot-treated RHM (Fig. 7). It is also worthy mentioning that, under our experimental conditions, inhibition of complex III activity by antimycin A in RLM had only a negligible stimulatory effect on ROS production, whereas Phyt and Ara exerted a potent effect (Fig. 6, panel B). These observations indicate that inhibition of the respiratory chain by FFA is not the only factor stimulating ROS production. This additional effect of FFA may facilitate the so-called “electron leak” due to changes in membrane fluidity. A more detailed analysis of the mechanism(s) by which FFA hamper electron transport and thus increase ROS production is, however, beyond the aims of this work.

The question can be, however, asked whether other ways of this increase are possible. One of them could be depletion of the mitochondrial glutathione pool. Reduced glutathione removes mitochondrially generated H\(_2\)O\(_2\) in a reaction catalysed by...
matrix glutathione peroxidase. In turn, the reduced form of glutathione is regenerated by the operation of glutathione reductase that utilizes NADPH as electron donor [56]. The latter compound is maintained in its reduced form due to the functioning of inner membrane-bound transhydrogenase utilizing NADH as reductant. This reaction is endergonic and depends on Δψm as the energy source [57]. Thus, decrease of Δψm by FFA due to their protonophoric action (mild uncoupling) might affect as well the scavenging ability of the mitochondrial matrix and increase ROS generation. In order to examine whether this might be the case, we investigated the effect of FFA on ROS production in glutathione-depleted mitochondria. As expected, glutathione depletion by itself resulted in a moderate increase of ROS generation. Nevertheless, this generation was further strongly increased by Phyto and, to a smaller extent, by Ara (Fig. 4). This result indicates that the increase of ROS production induced by FFA, similarly as that induced by antimycin A, was due to blocking the respiratory chain rather than to decreasing the scavenging action of the glutathione system. This conclusion is further reinforced by a correlation observed between stimulation of ROS generation and inhibition of complex III activity (Fig. 6).

In conclusion, the present results provide strong evidence that, in mitochondria functioning in FET mode, FFA boost ROS production by inhibiting the electron transport rather than decrease ROS generation due to their mild uncoupling action as proposed by the Skulachev’s group [7,15,16].

A different situation occurs in case of RET. In this situation high production of ROS is related to a high degree of the reduction of complex I that is, in turn, coupled to high Δψm. Any agents preventing RET, like inhibitors of the respiratory chain between complexes I and III (e.g., Rot), or protonophores (e.g. FCCP) could be expected to drastically diminish ROS production. In fact, the latter mechanism operates in case of low, micromolar, concentrations of Pal, Ole, Phyt and Ara, which appeared as potent inhibitors of ROS generation, with IC50 of about 5 nmol/mg protein (Fig. 2). In analogy, short-chain ceramides [53] and N-acyethanolamines [54] which are probably not protonophores by themselves but increase proton conductance of the inner mitochondrial membrane by some other mechanism(s), also inhibit ROS production based on RET.

In this point it could still be discussed whether FFA decrease Succ-dependent ROS production due to a moderate depolarisation of the inner membrane (FCCP-like effect) or to inhibition of RET by a direct interference with the respiratory chain (rotenone-like effect). However, reactivation of the Pal-suppressed ROS production by CAT clearly indicates that FFA suppress ROS generation in RET by their protonophoric activity (Fig. 2C).

In conclusion, with exception of RET-associated ROS production, FFA increase ROS generation driven by NAD+-linked substrates in heart and liver mitochondria of rat.

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