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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.

Keywords: Reactive oxygen species (ROS); Mitochondria; Fatty acid; Respiratory chain; Uncoupling; ADP/ATP carrier

1. Introduction

Generation of "reactive oxygen species" (ROS) by mitochondria was first described by Chance and co-workers [1–3]. Superoxide ($O_2^{\bullet-}$) 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 $O_2^{\bullet-}$ generation is attributed to ubisemiquinone as electron donor [3,6,7], the site of complex I-associated $O_2^{\bullet-}$ 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, $O_2^{\bullet-}$ is released from complex I to the matrix side of the inner membrane, whereas complex III releases $O_2^{\bullet-}$ to both sides of the inner membrane [12–14]. $O_2^{\bullet-}$ is rapidly converted to H_2O_2 by the Mn-containing superoxide dismutase (inside mitochondria) or the Cu,Zn-containing superoxide dismutase (intermembrane space and cytosol) [5]. H_2O_2 is decomposed by catalase or reduced to H_2O 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; $\Delta\psi_m$, 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 ($\Delta\psi_m$), 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 $\Delta\psi_m$. (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 10–12 $mg \times ml^{-1}$. 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 $mg \times ml^{-1}$. 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 KH_2PO_4, 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 (Oroboros Oxygraph®, Bioenergetics and Biomedical Instruments, Innsbruck, Austria).

2.3. ROS generation

ROS generation was estimated as the release of H_2O_2 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 $\times ml^{-1}$) were incubated in the standard incubation medium supplemented with 5 μM Amplex Red plus horseradish peroxidase (2 $U \times ml^{-1}$) to detect H_2O_2 and Cu,Zn-superoxide dismutase (2 $U \times ml^{-1}$) for quantitative conversion of released $O_2^{\bullet-}$ into H_2O_2. 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 H_2O_2. Addition of arachidonic or phytanic 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 Ewards [38]. Before measuring enzymatic activity, mitochondria were