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REVIEW

E. N. Mokhova

Involvement of Mitochondrial Inner Membrane Anion Carriers in the Uncoupling Effect of Fatty Acids

E. N. Mokhova* and L. S. Khailova

Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (7-095) 939-0338; E-mail: mokhova@genebee.msu.su

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Abstract—This paper considers stages of the search (initiated by V. P. Skulachev) for a receptor protein for fatty acids that is involved in their uncoupling effect. Based on these studies, mechanism of the ADP/ATP antiporter involvement in the uncoupling induced by fatty acids was proposed (Skulachev, V. P. (1991) *FEBS Lett.*, **294**, 158-162). New data (suppression by carboxyatractylate of the SDS-induced uncoupling, pH-dependence of the ADP/ATP and the glutamate/aspartate antiporter contributions to the uncoupling, etc.) led to modification of this hypothesis. During discussion of the uncoupling effect of fatty acids caused by opening of the Ca^{2+}-dependent pore, special attention is given to the effects of carboxyatractylate added in the presence of ADP. The functioning of the uncoupling protein UCP2 in kidney mitochondria is considered, as well as the diversity observed by us in effects of 200 μM GDP on decrease in $\Delta\psi$ under the influence of oleic acid added after H_2O_2 (in the presence of succinate, oligomycin, malonate). A speculative explanation of the findings is as follows: 1) products of lipid and/or fatty acid peroxidation (PPO) modify the ADP/ATP antiporter in such a way that its involvement in the fatty acid-induced uncoupling is suppressed by GDP; 2) GDP increases the PPO concentration in the matrix by suppression of efflux of fatty acid hydroperoxide anions through the UCP (Goglia, F., and Skulachev, V. P. (2003) *FASEB*, **17**, 1585-1591) and/or of efflux of PPO anions with involvement of the GDP-sensitive ADP/ATP antiporter; 3) PPO can potentiate the oleate-induced decrease in $\Delta\psi$ due to inhibition of succinate oxidation.

Key words: fatty acids, uncoupling, uncoupling proteins, anion carriers, ADP/ATP antiporter, GDP, mitochondria, peroxidation

The concept of noncoupled oxidation proposed by V. P. Skulachev suggests that in intact mitochondria the degree of coupling of respiration and oxidative phosphorylation can vary. Some physiological states associate with increase in mitochondrial noncoupled respiration (noncoupled with phosphorylation) [1-3]. Endogenous long-chain fatty acids that have the uncoupling effect are physiologically important activators of noncoupled oxidation. Fatty acids mediate the thermoregulatory uncoupling that markedly contributes to an increase in heat production in cases of short-term cold exposure of warm-blooded animals [2-4]. Uncoupling by fatty acids can suppress production of toxic ROS in mitochondria [3, 5, 6] and play an important role during other physiological and pathological processes accompanied by accumulation of fatty acids.

The uncoupling effect of long-chain fatty acids was discovered about fifty years ago [7], but the mechanism of this effect became clear only in the late 1970s. In mitochondria of brown fat tissue an uncoupling protein thermogenin was discovered that is responsible for the transmembrane transport of protons in the presence of low concentrations of fatty acids, and purine nucleotides inhibit this process ([8, 9] and references therein).

Even before and especially after the discovery of thermogenin, Skulachev recommended looking for a similar protein in mitochondria of skeletal muscles, and he attracted the attention of our group to some proteins, including the ADP/ATP antiporter. In experiments for more than a year, A. P. Agureev found that pre-addition to mitochondria of skeletal muscle of the ADP/ATP antiporter inhibitor atractylate or substrates of this anti-

Abbreviations: ROS) reactive oxygen species; CAtr) carboxyatractylate; FCCP) carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; PPO) products of fatty acid and/or lipid peroxidation; HNE) 4-hydroxy-2-nonenal; SDS) sodium dodecyl sulfate; UCP) uncoupling proteins; $\Delta\psi$) transmembrane electric potential difference across the mitochondrial inner membrane.

* To whom correspondence should be addressed.

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porter decreased the uncoupling effect of low concentrations of palmitic acid, i.e., decreased the respiration stimulated by fatty acids in the presence of the oxidation substrate and oligomycin. Although the effects were rather weak, it was suggested that the ADP/ATP antiporter, additionally to its main function (the exchange of intramitochondrial ATP for the extramitochondrial ADP) should be also involved in the uncoupling effect of fatty acids (A. P. Agureev and E. N. Mokhova, unpublished data). Continuing these experiments, A. A. Andreev used a powerful quasi-irreversible inhibitor of the ADP/ATP antiporter, CAtr. He showed that this inhibitor significantly decreased the stimulation of respiration and decrease in $\Delta\psi$ under the influence of palmitic acid but not of the uncoupler FCCP. Based on these findings and other experimental data [10, 11], it was suggested that the ADP/ATP antiporter should be involved in the uncoupling effect of low concentrations of fatty acids. These results were corroborated in many laboratories abroad. New findings in experiments with mitochondria and model systems have confirmed the involvement of the ADP/ATP antiporter in the uncoupling induced by fatty acids ([2, 3] and references therein).

Skulachev supposed that mechanisms of thermogenin and the ADP/ATP antiporter involvement in the uncoupling effect of fatty acids should be the same, and that these proteins should promote the transmembrane transfer of fatty acid anions from the matrix into the intermembrane space, whereas protonated fatty acids should enter the matrix without the help of the proteins [12].

CAtr relatively weakly suppressed the uncoupling effect of palmitic acid in liver mitochondria. Therefore, many authors believed that in liver mitochondria fatty acids transported protons through the mitochondrial inner membrane without involvement of proteins [13], similarly to uncouplers or fatty acids which increase the proton conductivity of the bilayer phospholipid membrane ([14-16] and references therein). According to Skulachev, the weak coupling effect of CAtr in these mitochondria was due to involvement in the uncoupling and of other proteins. V. N. Samartsev supposed that such a protein could be represented by the aspartate/glutamate antiporter, and this hypothesis was confirmed experimentally. In particular, substrates and inhibitors of the aspartate/glutamate antiporter produced the coupling effect—they suppressed the uncoupling induced by palmitic acid [17, 18]. Note that the contributions of CAtr and glutamate (aspartate) to the coupling effect that reflect the contributions of the ADP/ATP and aspartate/glutamate antiporters to the uncoupling, respectively depended on some factors, in particular, pH of the incubation medium. At pH 7.2 the main contribution was given by glutamate, whereas at pH 7.8 it was mainly provided by CAtr (in the presence of 3 mM $MgCl_2$ in the incubation medium), and the sum of the coupling effect of CAtr and glutamate was constant (about 80%) [19]. Other anion carriers could also be involved in the uncoupling effect of fatty acids (references in [3]).

Experiments with SDS led to modification of the hypothesis describing the mechanism of the uncoupling effect of fatty acids. SDS is different from lauric acid only by the sulfate group instead of carboxyl. As a result, at neutral pH virtually all SDS exists as anions. Nevertheless, SDS stimulates mitochondrial respiration similarly to palmitic acid, but in 10-fold higher concentration. Especially important was the finding that the SDS-induced stimulation of respiration was sensitive to CAtr [20]. In a study of proton transport in liver mitochondria by the acid pulse method, Brustovetsky showed that both SDS and palmitic acid increased the proton permeability of the inner mitochondrial membrane, and CAtr suppressed both effects to the same degree [20]. Based on these experiments, both the ADP/ATP and aspartate/glutamate antiporters were suggested to be involved not only in the transport of fatty acid anions from the matrix through the mitochondrial inner membrane, but also in their protonation [3].

The mechanism of the uncoupling effect of fatty acids was unclear for a long time. This was essentially because some authors used mitochondria with a low content of Ca^{2+}, while the others used Ca^{2+}-loaded mitochondria (see review [21]). In the latter case, the uncoupling was coupled with swelling of the mitochondria. In 1976, Hunter, Haworth, and Southard discovered that oleic acid can induce in the mitochondrial inner membrane a nonspecific pore for low-molecular-weight substances [22]. This Ca^{2+}-dependent pore could be also induced by various other substances with different properties [22-24]. Afterwards, intensive studies on mechanism of the Ca^{2+}-dependent nonspecific uncoupling effect of fatty acids caused by opening of the Ca^{2+}-dependent pore were started in many laboratories ([25-31] and references therein).

Hunter and Haworth found that inhibitors of the ADP/ATP antiporter influenced the opening of the Ca^{2+}-dependent pore [32]. Later, it was found that the ADP/ATP antiporter should have the "*c*" conformation to provide for the pore opening. Many authors think that the ADP/ATP antiporter is a component of the complex of proteins that forms the pore ([33] and references therein).

Dedukhova showed that the specific and nonspecific uncoupling effects of fatty acids were very different concerning the CAtr effect on the respiration of liver mitochondria in the presence of ADP [25]. She successively added to the mitochondria (in the presence of succinate and rotenone) oligomycin, Ca^{2+} or EGTA, palmitic acid, ADP, and CAtr. In the Ca^{2+}-containing sample, CAtr stimulated (with a lag-phase) respiration of the mitochondria. The respiration was not stimulated by CAtr if palmitic acid was exchanged for the uncoupler FCCP

(palmitic acid and FCCP produce the same stimulation of the respiration in the EGTA-containing incubation medium) [25]. However, in the presence of EGTA, CAtr inhibited the respiration of mitochondria stimulated by palmitic acid, and this characterized the specific uncoupling effect of fatty acids [11].

Later, we often used this test with CAtr to discriminate the specific and Ca^{2+}-dependent uncoupling effects of fatty acids. The presence of EGTA in the incubation medium not always prevented the pore opening (in particular, if the mitochondria had already accumulated Ca^{2+} during isolation). The sensitivity to the specific pore inhibitor cyclosporin A could be strongly decreased, in particular, when the pore opening occurred under the influence of oxidative stress [23]. The swelling of mitochondria could be very weak if the pore opening occurred in the substate with a limited permeability in the medium without addition of KH_2PO_4. The decrease in $\Delta\psi$ induced by CAtr in the presence of ADP was observed not only when the classic pore was induced by fatty acids but also when the pore opening occurred in the substate with selective cation permeability ([31] and references therein).

It should be noted that the pore can open at different substates. Thus, at the "low conductivity" substate it is permeable for Ca^{2+} but not for sucrose [34]. In our experiments with liver mitochondria a consecutive addition of 5-10 μM Ca^{2+} and 5-10 μM myristic acid (in the presence of oligomycin) resulted in a decrease in the $\Delta\psi$ sensitive to cyclosporin A. It was interesting that the decrease in $\Delta\psi$ was not accompanied by increase in the respiration rate (at least within the first 3-5 min). Based on these and other data on effects of nigericin, CAtr, and cyclosporin A, it was suggested that, under conditions of our experiments, Ca^{2+} and fatty acid on the initial stage open the pore at the substate with the selective permeability for cations and the decrease in the $\Delta\psi$ sensitive to cyclosporin A should be caused by its conversion to the pH gradient owing to the electrogenic transport of cations into mitochondria ([31] and references therein).

During the last decade, proteins similar to thermogenin in properties have been discovered in mitochondria not only of brown fat tissue but also in mitochondria of other tissues. They are called uncoupling proteins (UCP). Fatty acids increased and purine nucleotide decrease the proton conductivity of membranes of proteoliposomes with incorporated UCP ([35, 36] and references therein), as earlier found for thermogenin.

Studies on functioning in mitochondria of the newly discovered uncoupling proteins are difficult because of their low concentration. When interpreting data of such investigations, Brand and colleagues discriminated the effects of UCP2 and UCP3, first, by their being prevented by GDP and the absence in mitochondria from UCP2 and UCP3 knockout mice. These authors concluded that in kidney and skeletal muscle mitochondria exogenous or endogenous superoxide increased the proton conductivity of the mitochondrial inner membrane through UCP ([37, 38] and references therein).

The molecular mechanism, physiological functions, and regulation of new UCPs are still under discussion. The involvement of these proteins in thermogenesis seems doubtful because of their extremely low contents in mitochondria (as compared to thermogenin). Many experimental data can be explained by the hypothesis of UCP involvement in antioxidant defense. The major part of superoxide in the cell is generated in the respiratory chain of mitochondria, and H_2O_2 and other ROS are produced from superoxide. Skulachev and colleagues [6] found that a small decrease in $\Delta\psi$ (e.g., caused by fatty acids) sharply lowered the rate of H_2O_2 production. Despite their low concentrations, new UCPs could decrease the ROS generation if under the experiment conditions their contribution to the proton conductivity of the mitochondrial inner membrane was comparable with the total contribution of other anion carriers involved in the uncoupling.

According to a hypothesis of Goglia and Skulachev [39], UCPs are involved in the antioxidant defense also in another way: they translocate fatty acid hydroperoxide anions from the matrix into the intermembrane space and thus protect the mitochondrial DNA and other matrix proteins vitally important for the cell against damage by PPO. In [40, 41] PPO are considered to induce the proton conductivity through UCP.

The question of the UCP content in some tissues is still unsettled. The evaluation of the UCP content in mitochondria by amount of the tightly bound labeled GTP (K_d < 0.4 μM) gives the UCP concentration in kidney mitochondria approximately fourfold higher than in liver mitochondria [35]. Other authors determined the UCP2 content by binding to specific antibodies and concluded that kidney mitochondria are virtually free of UCP2, although they contain low concentrations of UCP2 mRNA [42]. These authors failed in reproducing the earlier experimental data on UCP2 activation by superoxide [37].

Such a variety of results of similar experiments could be caused by differences in physiological state of the animals, procedures of mitochondria isolation, etc. As an alternative explanation, GDP was suggested to inhibit the activity not only of UCPs but also of the ADP/ATP antiporter, the properties of which were changed under the influence of PPO.

According to [40], the uncoupling effect of HNE (a PPO) in kidney mitochondria was equally strongly inhibited by either GDP or CAtr. This indicated that UCP2 and the ADP/ATP antiporter were involved in the uncoupling consecutively but not in parallel. Another speculative explanation: HNE changed properties of the ADP/ATP antiporter in such a way, that GDP began to suppress its involvement in the uncoupling; normally, this is the effect of low concentrations of ADP [11].

We studied the decrease in $\Delta\psi$ in kidney mitochondria under the influence of oleic and palmitic acids (in the presence of oligomycin and malonate, with succinate as the oxidation substrate in the presence of rotenone), and special attention was given to the effect of the previously added GDP. The isolation and incubation media were similar to those described in [37] (nigericin was omitted). The effect of oleic acid on lipid peroxidation is not simple. On one hand, it binds the free iron and thus could suppress the iron-induced lipid peroxidation [43]. On the other hand, oleic acid itself could be involved in lipid peroxidation reactions and increase the production of PPO, which could variously affect mitochondria ([44] and references therein).

L. S. Khailova found that 200 μM GDP added before mitochondria either decreased (as in [37]) or increased the oleic acid-caused decrease in $\Delta\psi$ depending on the experimental conditions. The effects of GDP became more pronounced if mitochondria were washed and suspended in media with a decreased concentration of EGTA (from 2 mM to 50 μM) and addition of 1 mM H_2O_2 preceded the addition of oleic acid. In some experiments, GDP lowered the oleic acid-induced decrease in $\Delta\psi$ in the beginning of the experiment, but the effect of GDP changed to the opposite if the mitochondria were stored for a long time. In some experiments, GDP was ineffective [45]. The GDP-induced increase in the dissipation of $\Delta\psi$ was not caused by opening of the Ca^{2+}-dependent pore, because addition of CAtr after oleate failed to cause the further decrease in $\Delta\psi$ but in most of samples resulted in its increase, i.e., displayed a coupling effect.

According to published data, GDP inhibited the proton conductivity through UCP induced by fatty acids (or PPO), and this resulted in increase in $\Delta\psi$, which was lowered under the influence of these substances [37, 38, 40, 41]. GDP could strongly increase the concentration of PPO due to suppression of transfer of fatty acid peroxides from the matrix with involvement of UCP2 [39] and/or with involvement of the ADP/ATP antiporter, if it had acquired the sensitivity to GDP under the influence of PPO. We think that the change in direction of the GDP effect (potentiating the decrease in $\Delta\psi$) is caused by such a strong growth in the PPO content that it decreases $\Delta\psi$ due to inhibition of succinate oxidation. In experiments with liver mitochondria, it was earlier found that the uncoupling effect of linoleic acid PPO was stronger than the effect of the initial linoleic acid, and the higher concentrations of PPO inhibited succinate oxidation and decreased the rate of dichlorophenolindophenol reduction by succinate [46].

The above-mentioned interpretation of the published data and our findings is rather speculative, and experiments are to be continued. However, it is difficult to record $\Delta\psi$ and respiration of the same fraction of mitochondria. Lipophilic cations (their distribution is usually used for evaluation of $\Delta\psi$) are mainly accumulated in the fraction of mitochondria with the highest $\Delta\psi$, and the observed effects represent the properties only of this fraction. The rate of oxygen consumption characterizes the respiratory activity averaged over all fractions. Studies of UCPs in mitochondria are only beginning.

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