Fatty acid circuit as a physiological mechanism of uncoupling of oxidative phosphorylation

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Free fatty acids, natural uncouplers of oxidative phosphorylation, are shown to differ from artificial ones in that they fail to increase conductance of phospholipid bilayers which are permeable for the protonated form of fatty acids but impermeable for their anionic form. Recent studies have revealed that uncoupling by fatty acids in mitochondria is mediated by the ATP/ADP antiporter and, in brown fat, by thermogenin which is structurally very similar to the antiporter. It is suggested that both the ATP/ADP antiporter and thermogenin facilitate translocation of the fatty anions through the mitochondrial membrane.

Fatty acid; Uncoupling; ATP/ADP antiporter; Thermogenin

1. FATTY ACID-MEDIATED THERMOREGULATORY UNCOUPLING

In 1960 we proposed that uncoupling of oxidative phosphorylation is a mechanism of the urgent heat production by warm-blooded animals when the ambient temperature strongly decreases [1] (see also [2]). This proposal was based upon our observation that 15 min cold exposure of pigeons previously adapted to cold stresses results in a 6-fold decrease in the P/O ratio in the breast muscle mitochondria [1,2]. Such an effect was then reproduced in the muscle mitochondria of mouse [3] and of fur seal [4].

The uncoupling was shown to be abolished by adding serum albumin [2,4]. This fact could be explained suggesting that fatty acids are involved in the above effect [5] since (i) free fatty acids uncouple oxidation and phosphorylation in mitochondria as first described by Pressman and Lardy [6], and (ii) serum albumin binds fatty acids and recouples mitochondria [5]. The above suggestion was confirmed by our experiments showing that the short-term cold exposure increases the level of fatty acids both in muscle in situ and in mitochondria isolated from muscle of the cold-exposed animal. Fatty acids extracted from mitochondria of the cold-exposed

animals and then added to mitochondria from the non-exposed ones were found to cause uncoupling [7].

The idea of thermoregulatory uncoupling mediated by fatty acids was later confirmed and extended in numerous studies on brown fat, the mammalian tissue specialized in additional heat production under cold conditions (for reviews, see [8-12]). In the brown fat mitochondria, the uncoupling protein (the other name, thermogenin) has been discovered [13,14]. This protein increases H^+ conductance of the brown fat mitochondrial membrane.

The uncoupling activity of thermogenin was shown to require free fatty acids [11,12,15-17]. It is not clear yet how fatty acids activate thermogenin as well as how they uncouple in tissues other than brown fat where there is no thermogenin. It seems obvious that fatty acids cannot operate as well-known artificial protonophorous uncouplers such as trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP). In contrast to FCCP, which strongly increases the conductance of planar phospholipid bilayers and liposomes, fatty acids are almost without effect on the conductance of planar membrane and cytochrome oxidase proteoliposomes [18].

Such an inefficiency of fatty acids as protonophores in model systems can be easily explained by the fact that they can traverse the phospholipid membrane only in their protonated form (RCOOH) [18] whereas the anion (RCOO⁻) tends to occupy a position in the lipid/water interface with the carboxylate group facing the water and the hydrocarbon 'tail' penetrating into the membrane core. In the case of the FCCP anion, the negative

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Abbreviations: Catr, carboxyatractyloside; FCCP, trifluoromethoxycarbonylcyanide phenylhydrazone

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charge is strongly delocalized which facilitates diffusion of the anion through the interface [19].

2. FATTY ACID CIRCUIT HYPOTHESIS

In 1988 I suggested [19] that in mitochondria there are proteins which allow the anionic forms of fatty acids to traverse the mitochondrial membrane. The proteins in question were supposed to be thermogenin in brown fat and the ATP/ADP antiporter in other tissues (Fig. 1). Within the framework of this scheme, the ATP/ADP antiporter, in addition to its main function, i.e. the translocation of adenine nucleotide anions, can also transport fatty acid anions. As to thermogenin, it should be regarded as a derivative of the ATP/ADP antiporter, which has lost the main function but retains the additional one.

The following observations seem to indicate that the ATP/ADP antiporter is somehow involved in the fatty acid-induced uncoupling.

(i) When studying muscle and liver mitochondria, we have found that uncoupling induced by low concentrations of fatty acids is suppressed by CAtr, the most potent and specific inhibitor of the ATP/ADP antiporter. The K_i values for CAtr inhibition (1) of oxidative phosphorylation and (2) of the fatty acid-stimulated respiration in the presence of the H^+-ATP-synthase inhibitor, oligomycin, proved to be similar [18,20–23].

(ii) The CAtr effect is accompanied by a membrane potential increase [18] and by a decrease in the H^+ conductance of the mitochondrial membrane [22].

(iii) Other inhibitors of the ATP/ADP antiporter such as atractyloside, bongkrekic acid and pyridoxal phosphate, as well as its substrate, ADP, are also inhibitory for the fatty acid-induced uncoupling, being, however, less effective than CAtr [18,22].

(iv) Uncoupling by FCCP is insensitive to CAtr [18,22].

In this context, it is noteworthy that fatty acids inhibit the ATP/ADP antiporter at low concentrations. Bongkrekic acid, a specific ATP/ADP antiporter inhibitor, represents a long-chain fatty acid with three carboxylate groups [19]. Also CAtr and atractyloside are hydrophobic carboxylates.

Several pieces of evidence indicate close structural and functional relations of the ATP/ADP antiporter and thermogenin.

(A) The two proteins are of the similar molecular mass, sequence, secondary structure, transmembrane arrangement and domain composition [24–26].

(B) Both of them are formed without the stage of a larger precursor, a very unusual situation for mitochondrial inner membrane proteins encoded by the nuclear genes [19].

(C) Both of them can interact with purine nucleotides [24] and fatty acids [19]. The interactions occur in such a way that fatty acids induce uncoupling whereas purine nucleotides cause recoupling.

(D) Not only the ATP/ADP antiporter but also thermogenin can be regarded as anion carrier since thermogenin increases the membrane permeability for Cl^- [27–29].

It should be stressed that features (B) and (C) clearly distinguish the ATP/ADP antiporter and thermogenin from the mitochondrial phosphate and dicarboxylate carriers which also resemble these proteins in the sequence and domain structure [26].

Suggesting that the ATP/ADP antiporter is competent in translocating not only nucleotide anions but also fatty acid anions, we face the problem why this carrier, discriminating between e.g. ADP and GDP, fails to discriminate between ADP and fatty acid. To overcome this difficulty, it seems reasonable to assume that the high nucleotide specificity of the ATP/ADP antiporter is a property of the gate (binding site) required to interact with **hydrophilic** anions whereas **hydrophobic** fatty acid anions can be effectively bound to the anion-translocating part of the protein with no specific gate involved.

Within the framework of the above reasoning, thermogenin may be regarded as a product of the ATP/ADP antiporter evolution. Thermogenin still binds nucleotides but fails to do this in the proper way and therefore cannot transport bound nucleotides through the membrane. At the same time, thermogenin retains the anion translocating machinery and, hence, can transport fatty acid anions which do not require the gate nucleotide binding site to be translocated. It is noteworthy that in thermogenin, the nucleotide binding site is of lower specificity than in ATP/ADP antiporter (it interacts not only with adenine but also with guanine nucleotides) [10].
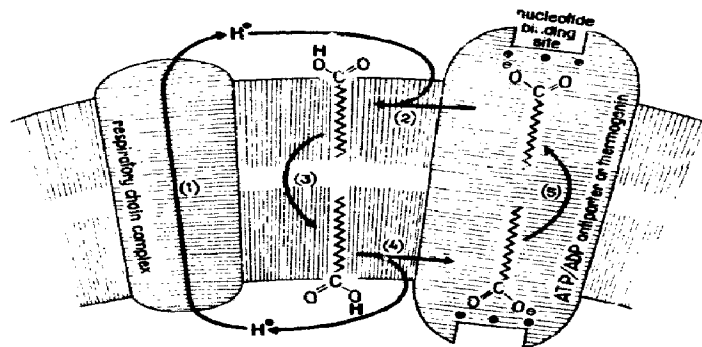

Fig. 1. The fatty acid circuit mediated by the ATP/ADP antiporter and thermogenin results in the dissipation of the respiration-generated protonic potential. (1) The respiratory chain pumps H^+ from the mitochondrial matrix to the extramitochondrial space; (2) H^+ protonates fatty acid on the outer surface of the inner mitochondrial membrane; (3) the protonated fatty acid traverses the phospholipid bilayer of the membrane; (4) the fatty acid is deprotonated on the inner surface of the membrane; and (5) the formed fatty acid anion, for which the bilayer is impermeable, is outwardly translocated by the ATP/ADP antiporter or thermogenin.

3. RECENT OBSERVATIONS IN FAVOUR OF THE HYPOTHESIS

Quite recently our data were reproduced and extended in Germany by Schönfeld [23] who showed that the efficiency of fatty acid as the CAtr-sensitive uncoupler is proportional to the ATP/ADP antiporter content in mitochondria of different tissues (heart muscle > kidney > liver).

Our scheme predicts that both the ATP/ADP antiporter and thermogenin should be rather non-specific towards hydrophobic anions. In 1990 this suggestion was directly proved. We have shown [22] that not only fatty acids but also small amounts of artificial anionic detergents such as dodecyl sulfate and cholate can cause the CAtr-sensitive uncoupling in rat liver mitochondria. Under the same conditions, uncoupling by a cationic detergent (cetyltrimethyl ammonium) or by a non-ionic detergent (Triton X-100) was CAtr-resistant. Low concentrations of dodecyl sulfate and cholate inducing a CAtr-sensitive release of the respiratory control were found to increase specifically the CAtr-sensitive H^+ permeability of mitochondria. The uncoupling concentrations of cholate and dodecyl sulfate were much lower than those causing the non-specific damage of mitochondria but much higher than the uncoupling concentrations of palmitate.

Similar features were shown to be inherent in thermogenin. According to Rial et al. [30] and Klingenberg [16], not only wide-spread natural fatty acids like palmitate, stearate and oleate, but also carboxylic acids with a shorter hydrocarbon chain are still competent (but at higher concentrations) in activating the thermogenin-mediated H^+ conductance. Recently Garlid showed [27] that hexane sulfonate can substitute for palmitate in activating the H^+ conductance via thermogenin, the half-maximal concentration of hexane sulfonate being more than 10^3 times higher than that of palmitate. The effect of hexane sulfonate looks quite similar to that of dodecyl sulfate (see above).

The most important observation made by Jezek and Garlid [27,31] in 1990 is that they demonstrated **the purine nucleotide-sensitive transport of hexane sulfonate** to the brown fat mitochondria and thermogenin proteoliposomes. Further studies showed that not only hexane sulfonate but also a large group of monovalent, monopolar anions can be transported by thermogenin. The list of transportable anions includes alkylsulfates, benzenesulfonate, oxohalogenides, hypophosphate, hexafluorophosphate and pyruvate. The polar group must not be blocked by alkyl or aryl substituents.

It is interesting that non-specificity of the thermogenin-induced conductance was already recognized by its first students, Nicholls and Lindberg [28], who found that brown fat mitochondria are permeable not only to H^+ but also to Cl^-. Garlid and co-workers [27,29,31] reconstituted Cl^- transport in thermogenin proteoli-

posomes and showed that, unlike in the case of H^+ transport, fatty anions are not necessary for this process. Rather they seem to compete with Cl^- for thermogenin. The rate of Cl^- transport proved to be much lower than that of hexane sulfonate and even of butane sulfonate. Generally the rates of transport (as well as K_i values for purine nucleotide inhibition) are shown to increase with the length of the alkyl residue. Unfortunately, the method of transport rate measurements (the swelling of the brown fat mitochondria incubated with 0.05 M sodium salts of the anions) did not allow for the study of long-chain fatty acids. However, the above mentioned dependence of the transport rate upon the length of the hydrocarbon chain strongly suggests that fatty acid anions should be effectively transported by thermogenin. Combining this suggestion with the very fact that protonated fatty acids can easily traverse the membrane, one may conclude that at least a portion of the thermogenin-mediated H^+ conductance is due to fatty acid circulation. The minimal hypothesis consists in that this is the only mechanism of uncoupling by thermogenin.

4. SOME APPARENT DIFFICULTIES

There is an opinion that H^+ and Cl^- are transported via two different pathways both mediated by thermogenin [32]. According to our scheme, the difference in the mechanism of H^+ and Cl^- translocations consists in that H^+ conductance involves circulation of a weak acid, natural fatty acids being the most effective, whereas Cl^- does not require such a circulation. Nevertheless, there is no reason to speak about two pathways, one for H^+ and the other for Cl^- since it is assumed that both Cl^- and fatty anions compete for the same anion-carrying groups or anionic channel in thermogenin. An observation that H^+ transport is sensitive to organomercurials whereas the Cl^- transport is not [32], seems hardly conclusive since organomercurials such as mersalyl form complexes with Cl^- [33] and induce Cl^- permeability [34]. An alternative possibility is that the pathways of hydrophilic Cl^- and hydrophobic fatty acid anions towards the anion-carrying sites in thermogenin are different, the latter being specifically blocked by SH-reagents.

On the face of it, there is a difficulty in our scheme in the assumption of the existence of the protonated forms of alkyl sulfonates and alkyl sulfates at neutral pH. This assumption is necessary to explain how e.g. dodecylsulfate uncouples rat liver mitochondria in the CAtr-sensitive fashion [22]. In fact, the pK_a values of these anions are so strongly shifted to acidic pH values that the concentration of their protonated forms at neutral pH is negligible. However, such a reasoning is valid for the bulk water phase, not for the membrane/water interface. According to Sankaram et al. [35], the pK_a values of fatty anions in the interface are very much

higher than in aqueous solution. Thus, the pK_a of stearate in the dimyristoyl phosphatidylglycerol liposomes is as high as 8.0 (in aqueous solution, 5.0). The pK_a value could be further increased up to 9.6 when some proteoliposomes were used instead of liposomes.

The non-specificity of the porters to the structure of transported anions seems one more surprising feature of the systems. However, it should be stressed that e.g. thermogenin non-specificity is, most probably, not manifested under physiological conditions since millimolar concentrations of purine nucleotides, which are always present in the cell, are quite sufficient to block translocation of the anions which are less hydrophobic than natural fatty acids.

In this context, it is apt to mention the general anion carrier postulated in mitochondria by Beavis et al. [36,37] and the observation by Krämer's group that the SH reagent-modified ATP/ADP antiporter in proteoliposomes becomes competent in the ATP anion uniport [38].

5. PHYSIOLOGICAL IMPLICATION

There is no doubt that the fatty acid and thermogenin-mediated uncoupling is involved in the regulatory heat production by brown fat (for reviews, see [11,19]). As to other tissues, involvement of fatty acids in uncoupling under cooling seems also highly probable (see above and ref. [19]) but the precise mechanism of their action should be systematically studied. The ATP/ADP antiporter-mediated uncoupling is the simplest possibility which was directly proved in our group at least in one case namely during arousal of ground squirrels after hibernation [39]. In the oligomycin-treated heart muscle and liver mitochondria from arousing animals, CAtr was shown (i) to decrease the respiration rate, (ii) to increase the membrane potential and (iii) to lower the rate of the membrane potential discharge after addition of cyanide. Serum albumin effectively substituted for CAtr so that CAtr added after albumin had no effect. Arousal was also found to be accompanied by an increase in the free fatty acid content both in the heart muscle and in the mitochondria isolated from this muscle. The fatty acid concentration increased and the CAtr-sensitive uncoupling disappeared when the body temperature of the ground squirrel reached 37°C. The simplest way to recouple the fatty acid-uncoupled mitochondria would be to oxidize the excess of the mitochondrial free fatty acids.

6. SOME OTHER HYPOTHESES

An alternative explanation of the fatty acid-mediated uncoupling would be that fatty acids serve as allosteric activators of the H^+ conductance through the ATP/ADP-antiporter and thermogenin.

Klingenberg mentioned that 'fatty acids may form a

micellar H^+ buffer near or in the translocation channel of uncoupling protein' [40]. If this is the case, the question arises whether such an effect is the only role of fatty acids in the uncoupling, or the local pH buffering is supplemented by circulation of fatty acids as shown in Fig. 1.

Jezek and Garlid [31] considered five possible patterns of the thermogenin mechanism, namely (1) anion channel, (2) anion channel with a hydrophobic barrier for anions, (3) separate H^+ and anion pathways, (4) anion channel which provides a site for allosteric fatty acid activation of H^+ uniport and (5) thermogenin as a member of the family of mitochondrial anion porters. Some of these models, like that of Klingenberg, can be combined with our fatty acid circulation hypothesis whereas others cannot. If the latter were the case, the most probable situation would be that two mechanisms of thermogenin uncoupling co-exist, one postulated by Jezek and Garlid and another shown in Fig. 1. Such an assumption is a direct consequence of observations made by the same authors that hexane sulfonate (i) is translocated via thermogenin, (ii) replaces fatty acids in activation of the thermogenin-mediated H^+ conductance and (iii) both these properties are inherent in other monovalent anions, increasing with the increase in the length of the hydrocarbon 'tail'. The above item strongly suggests that natural fatty acids belong to the family of thermogenin-transported anions.

There are some indications that the thermogenin or ATP/ADP antiporter-mediated uncoupling is not the only mechanism of dissipation of the respiratory energy by free fatty acids. At least in vitro, one more mode of uncoupling, also activated by fatty acids, has been described. It requires Ca^{2+} and includes a cascade of several processes such as phospholipid hydrolysis, peroxidation and some others [18,41,42]. It is, however, not clear whether this system is operative in vivo under conditions of the thermoregulatory heat production. Among other possible fatty acid effects which can result in uncoupling we may mention (i) ΔpH dissipation due to diffusion of the protonated fatty acids via phospholipid part of the membrane, (ii) increase in dielectric constant of the membrane system which might lower the contribution of the local electric field to the energy coupling, (iii) transport of the fatty anions by proteins other than the ATP/ADP antiporter and thermogenin. One of these effects may be responsible for the fatty acid uncoupling activity in chloroplasts [43].

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