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Mitochondrial Uncoupling Proteins – Facts and Fantasies

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Summary

Instead of a comprehensive review, we describe the basic undisputed facts and a modest contribution of our group to the fascinating area of the research on mitochondrial uncoupling proteins. After defining the terms uncoupling, leak, protein-mediated uncoupling, we discuss the assumption that due to their low abundance the novel mitochondrial uncoupling proteins (UCP2 to UCP5) can provide only a mild uncoupling, i.e. can decrease the proton motive force by several mV only. Contrary to this, the highly thermogenic role of UCP1 in brown adipose tissue is not given only by its high content (~5 % of mitochondrial proteins) but also by the low ATP synthase content and high capacity respiratory chain. Fatty acid cycling mechanism as a plausible explanation for the protonophoretic function of all UCPs and some other mitochondrial carriers is described together with the experiments supporting it. The phylogenesis of all UCPs, estimated UCP2 content in several tissues, and details of UCP2 activation are described on the basis of our experiments. Functional activation of UCP2 is proposed to decrease reactive oxygen species (ROS) production. Moreover, reaction products of lipoperoxidation such as cleaved hydroperoxy-fatty acids and hydroxy-fatty acid can activate UCP2 and promote feedback down-regulation of mitochondrial ROS production.

Key words

Mitochondria • Mitochondrial uncoupling • Uncoupling proteins • Fatty acids • Reactive oxygen species

Introduction

At the time of our department foundation (1991, *www.mitonet.cz*) it seemed that the research on the mitochondrial uncoupling protein-1 (UCP1) of brown adipose tissue (BAT) mitochondria cannot bring any new surprise. Yet in 1995 a second known uncoupling protein was found in plants (termed PUMP) and since 1997, even prior to human genome sequencing, the four new UCP isoforms (UCP2 to UCP5) were identified in humans or rodents. At the same time, mitochondria were recognized not only as simple energy generators, but as organelles regulating various physiological phenomena and playing the role of "decision makers" within the information cascade of apoptosis. Thus, on advent of mitochondrial physiology, new roles for regulated uncoupling are found and novel UCPs are implicated in numerous physiological and pathological phenomena. Their importance is clear from a simple list: prevention of reactive oxygen species (ROS) formation, prevention of atherosclerosis, one of etiologies of type-2 diabetes, participation in inflammation, body weight regulation, adaptive thermogenesis (including fever), and aging. There are only few reports on UCP4 and UCP5, but the research on UCP2 and UCP3, which has spread into many laboratories in the world, is so extensive that to

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cover this field by a review (Ježek and Garlid 1998, Ježek 2002) can provide only a "snapshot", which already becomes obsolete a year later. Hence, instead of providing here a comprehensive review, we describe the basic undisputed facts and a modest contribution of our group to this fascinating area of the research.

Uncoupling as an inherent part of mitochondrial physiology

Machinery of oxidative phosphorylation

In the mitochondrial matrix, eight enzymes of the citric acid cycle oxidize the acetyl group of acetyl-CoA (product of glycolysis and β-oxidation of fatty acids) to two molecules of CO_2 with the concomitant generation of three NADH molecules, one $FADH_2$, and one GTP (Pedersen 1999). Coenzymes NADH and $FADH_2$ are reoxidized passing electrons to the electron transport chain, consisting of four complexes in the inner mitochondrial membrane – Complex I (NADH-Coenzyme Q reductase, or NADH-dehydrogenase), Complex II (Succinate-Coenzyme Q reductase), Complex III (Coenzyme Q-cytochrome c reductase), and Complex IV (cytochrome c oxidase). The electrons passing through I-III-IV or II-III-IV complexes participate in oxidation/reduction of over 10 redox centers and finally four electrons reduce O_2 to H_2O in the Complex IV. The involved energy transformation allows components of Complex I, III and IV to pump H^+ across the inner mitochondrial membrane from the matrix to the intermembrane space. This creates an electrochemical H^+ gradient (called the proton motive force, Δp, when divided by Faraday constant and expressed in mV), which provides energy for ATP synthesis on the F_OF_1-H^+ATPase or ATP-synthase (Complex V). At least three H^+ are required to pass the F_O part of ATP-synthase (subscript O indicates inhibition by oligomycin) to synthesize one ATP. H^+ "flux" through the F_O part back to the matrix results in the rotation of γ-subunit of F_1 and induction of sequential conformation changes within the three α and three β subunits. This sequentially creates loose-binding (L), tight-binding (T) and open conformation (O) of three catalytic sites which coexist with other three noncatalytic sites of F_1. Thus energy-dependent conformation changes convert L to T, T to O and O to L. ADP and phosphate bind to L state and are released as ATP in the O state. The whole process is called the oxidative phosphorylation (OxPho). The efficiency in use of the formed H^+ gradient is called c*oupling*. However, the H^+ gradient is not consumed only by the ATP-synthase, but its electrical part (ΔΨ) is consumed by the ADP/ATP carrier and glutamate/aspartate carrier (due to one charge imbalance in exchange of their substrates) and the ΔpH part is consumed by the phosphate carrier and other carriers employing substrate-H^+ symport. Hence, the 100 % coupling would exist, if the whole OxPho machinery worked at the expense of the whole H^+ gradient formed. On the contrary, when any mechanism allows H^+ backflow to the matrix which bypasses the F_O part of ATP-synthase, the process is called *uncoupling*.

Uncoupling, leak, and uncoupling protein

Due to the so-called respiratory control, uncoupling usually leads to acceleration of respiration, since in many tissues and cell types the capacity of respiratory chain overcomes the capacity of ATP-synthase and other processes consuming the H^+ gradient. When these capacities match or the respiratory chain capacity is lower, the phosphorylating respiration (State III) is equal to uncoupled respiration caused by the artificial protonophores, (dinitrophenol, CCCP, FCCP etc). However, the typical situation is that uncoupled respiration is faster than the State III respiration.

Nevertheless, uncoupling should be considered as the inherent part of OxPho. Under normal conditions, a portion of the created H^+ gradient is consumed by the H^+ backflow to the matrix via non-protein membrane pores or protein/lipid interfaces and this is called an *H^+ leak* (Porter *et al*. 1996). On the other hand, when a protein pathway exists, which enables H^+ backflow to the matrix, we define such process as a *protein-mediated uncoupling*. It could proceed by any mechanism, but should not be part of the main OxPho machinery (ADP/ATP, phosphate and H^+ symport transport mechanisms are excluded).

Uncoupling of mitochondria was originally considered as an isolation artifact (Skulachev 1998). Since the discovery of brown adipose tissue (BAT) in 1961, it has been slowly recognized, that a factor that uncouples BAT mitochondria is an uncoupling protein (~1974). Protocols of mitochondrial isolation use albumin (BSA) to deplete fatty acids (FAs) and other compounds. Omitting BSA during isolation of plant mitochondria enabled demonstrations of re-coupling by BSA together with one of purine nucleotide di- or triphosphates (PNs) in potato tuber mitochondria (Ježek *et al*. 1996b). This is equal to re-coupling observed in BAT mitochondria. It shows that plant UCP behaves in the same way as UCP1 in BAT. The discovery of UCP2, expressed (although in minute amounts) in mitochondria of all human or

mammalian tissues, attracted a great attention of scientists of all biomedical fields (Ježek 2002) and the phenomena attributed to an unidentified leak may now be ascribed partly to highly regulated UCP2.

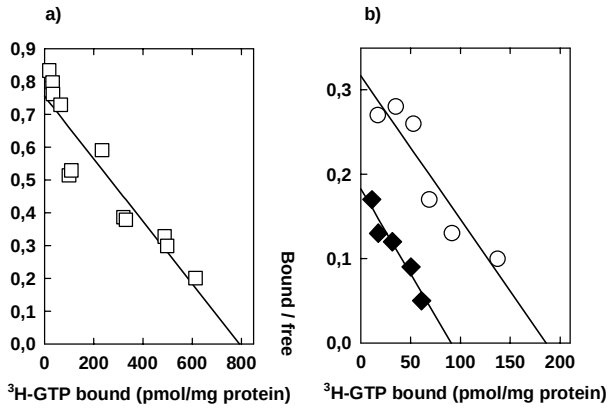

Fig. 1. ^{3}H-GTP binding: **a)** to isolated hamster brown adipose tissue mitochondria (□), **b)** to rat brain (◆), and rat spleen mitochondria (○). The Scatchard plots are shown with subtracted data for background, measured with 2.5 mM "cold" GTP. Derived number of binding sites corresponded to 792 pmol/mg protein, 91 pmol/mg protein and 186 pmol/mg protein for hamster BAT, rat brain, and rat spleen mitochondria, respectively, while the derived binding constants K_ds were 0.95 μM (i.e. for UCP1) in BAT mitochondria, 0.5 μM in rat brain (possibly UCP2, UCP4 and UCP5); and 0.53 μM in rat spleen mitochondria (UCP2).

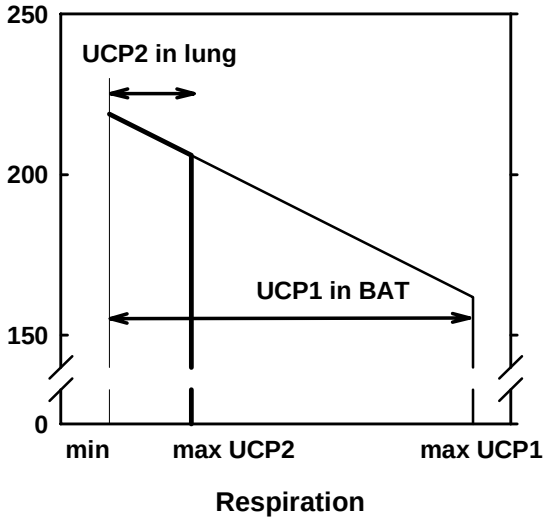

Fig. 2. Contribution of UCP1 to uncoupling in BAT mitochondria and UCP2 to uncoupling in lung mitochondria. Schema based on the experimental data for BAT mitochondria (Nicholls 1974), where UCP1 contribution to uncoupling causes increase in respiration from the coupled rate of 43 nmol O min^{-1} mg protein^{-1} to maximum 140 nmol O min^{-1} mg protein^{-1}. For 4.4-times less abundant UCP2 in rat lung mitochondria (Žáčková *et al.* 2003) maximum uncoupled rate would differ from the coupled rate only by 21.8 nmol O min^{-1} mg protein^{-1}, which is a fraction of the corresponding extent in BAT mitochondria. The related protonmotive force decrease would correspond to ~ 12.5 mV.

What uncoupling proteins can really provide

The physiological role of UCPs, mitochondrial uncoupling proteins, is determined not only by the amount of expressed protein but also by the extent of their activation. Thermogenesis in BAT cells is achieved by UCP1 accounting for ~5 % of the whole mitochondrial protein (800 pmol of dimeric UCP1 per mg protein, 1.6 nmols of 0.033 mg per nmol, see Fig. 1). Classic experiment (Nicholls 1974) shows that Δp relationship to the respiration rate for BAT mitochondria consists of a vertical line from ~166 mV to zero reflecting maximum respiration of 140 nmol O.min^{-1}.(mg protein)$^{-1}$ as limited by the dehydrogenase. The states with Δp between 166 mV and ~0 mV belong to uncoupled states. Partially coupled states above 166 mV up to the maximum coupled state at ~217 mV (respiration of 43 nmol O.min^{-1}.(mg protein)$^{-1}$) were reflected by a straight line showing the reciprocal proportionality between Δp and respiration. These states were experimentally established either by minute amounts of an uncoupler (FCCP) or by blocking UCP1 with GDP, BSA, or GDP plus BSA. Hence, the maximum extent of uncoupling, which UCP1 can achieve in BAT mitochondria, corresponds to ~55 mV (taken from a fit by equation R = 415 – 1.7. Δp, R is respiration, Nicholls 1974). However, since UCP2 is present according to our estimations (see expression of uncoupling proteins) in at least 4.4 times and >40 times lower amounts (*vs*. UCP1 in BAT) in lung mitochondria and mitochondria from unstimulated liver, respectively, one can calculate (Fig. 2) that maximum possible uncoupling given by UCP2 will be 12.5 mV and 1.4 mV, respectively. Feasibility of this estimation lies in the fact that V_{max} and K_m for UCP1, UCP2, and UCP3 are quite similar when activated by the same fatty acid (Žáčková *et al*. 2003, Jabůrek *et al*. 1999, Jabůrek and Garlid 2003). Note, however, that the calculated estimations are valid for maximum activated UCP2 (by FAs) at maximum released inhibitory regulators, such as PNs (Žáčková *et al*. 2003). Consequently, maximum uncoupling due to UCP2 function under physiological conditions will be of much smaller extent. For such an uncoupling, the term *mild uncoupling* has been introduced (Skulachev 1998, Ježek *et al*. 1998, 2001). Thermodynamical considerations showed that for an open system, the efficiency of a certain parameter is actually higher when the efficiency of coupling is slightly below one (Stucki 1980). In any case, the mild uncoupling slightly accelerates respiration and hence upstream metabolism, being beneficial for many physiological processes (Ježek 2002, Ježek *et al*.

1998, 2001) and suppresses radical formation. It also prevents establishment of the excessive H^+ gradient and situation when synthesized ATP would inhibit the respiratory chain. Mild uncoupling also provides a very low heat release with the regard to the state without uncoupling. Such "non-thermogenic" mild uncoupling given by UCP2 and UCP3 can have a long-term regulatory role (days, years) for body weight regulation. It is not known, whether any of UCPs besides UCP1 enables highly uncoupled states and if is surrounded in certain tissues by a suitable mitochondrial OxPho machinery which enables thermogenesis.

Indeed, in order to induce a sufficient heat release per min to raise the temperature of the given tissue, a substantial protein-mediated uncoupling must be induced, at least exceeding the uncoupling given by the H^+ leak. Heat release by the ordinary metabolism including the H^+ leak is called *obligatory* (or *basal) thermogenesis*. A*daptive thermogenesis* (or *regulatory)* is the heat release induced above this level, e.g. thyroid hormone-induced thermogenesis or fever (Janský 1995). *Non-shivering thermogenesis* in BAT is due to UCP1-mediated uncoupling activated by FAs (Ježek 1999, Nedergaard and Cannon 1992) and is given not only by the own uncoupling capacity (relative amount of UCP1), but also by the high respiratory chain capacity and the relatively low content of ATP-synthase.

Brown adipose tissue uncoupling protein-1 as the only thermogenic uncoupling protein?

Unique properties of brown adipose tissue

Brown adipose tissue (BAT) was recognized as an organ of nonshivering thermogenesis in newborns, cold- acclimated and hibernating mammals, and in overfed rodents (Nedergaard and Cannon 1992). The tissue is well suited for its thermogenic role, because BAT cells are loaded with mitochondria containing the tissue-specific UCP1; each cell is inervated, tissue has a rich microvasculature, and the majority of blood flow is passed through BAT (Ježek 1999). The presence of a relatively low ATP-synthase content in BAT mitochondria and their high capacity to oxidize fatty acids and α-glycerolphosphate and rich respiratory chain also causes that H^+ pumping has so high speed and capacity that the H^+ backflow into the matrix enabled by UCP1 is indeed thermogenic. UCP1 in BAT provides an efficient and regulated non-shivering thermogenesis to the whole body. When these unique properties of the tissue and mitochondria are not preserved, even UCP1 e.g. expressed in heart tissue does not cause any significant uncoupling or heat production (Bouillaud and Hoerter 2003). UCP1 expression and cell differentiation in pre-adipocytes is acutely regulated by norepinephrine via β_3 adrenergic receptor – the β_3 activation also leads to the initiation of the lipolytic cascade releasing FAs which directly activate UCP1, since simultaneously produced modulators prevent purine-nucleotide (PN) inhibition of UCP1.

Mechanism of uncoupling mediated by UCP1

Originally, the PN-inhibited H^+ translocation physically ongoing in the UCP1 structure became the "common dogma" in the field (Klingenberg 1990). This emerged in the concept of a "*local buffering*" by fatty acids (FAs, Klingenberg and Huang 1999), which assumes that ionized (anionic) FAs loosely associated with UCP1 participate in jumps of H^+ over an array of sites forming the H^+ translocation pathway. But no such array was identified in any UCP structure. Not all the authors have yet recognized (Gonzalez-Barroso *et al*. 1998) that the FA presence is essential for activating protonophoric function of UCP1, as we have definitively proven recently (Urbánková *et al*. 2003). A consensus on FA requirement became evident from earlier attempts to reconstitute UCP1 into liposomes (Strieleman *et al*. 1985a, Klingenberg and Winkler 1985, Ježek *et al*. 1990a, Murdza-Inglis *et al*. 1991, Winkler and Klingenberg 1992, 1994). The only contradictory fact was the finding that UCP1 transports monovalent unipolar anions (Ježek and Garlid 1990), halides (Nicholls and Lindberg 1973, Strieleman *et al*. 1985b, Ježek *et al*. 1989b, 1990b, Murdza-Inglis *et al*. 1991), non-physiological anions such as alkylsulfonates, monovalent phosphate analogs (Ježek and Garlid 1990), but also physiological ketocarboxylates such as pyruvate (Ježek and Borecký 1998). All anions, recognized on the basis of mitochondrial assays to be UCP1 substrates, were also found to be competitive inhibitors of Cl^- uniport (Ježek and Garlid 1990). This competition includes FAs as well (Ježek *et al*. 1994, Garlid *et al*. 1996). The mutual competition of FAs and anions has led us to develop the concept of a "docking site", a common part of binding sites for anions and FAs. This concept had preceded the fatty acid cycling hypothesis (Skulachev 1991, 1998), predicting that not only UCP1, but also carriers such as the ADP/ATP carrier, can mediate a uniport of FA anion, while the neutral (protonated) FA

subsequently returns back across the lipid bilayer and carries H^+. During the years we have brought the three lines of support for the FA cycling mechanism (Garlid *et al*. 1998, 2000, 2001, Ježek 1999, Ježek *et al*. 1998, 2001): i) mutual competition between FAs and anions; ii) undecanesulfonate behavior, and iii) existence of so called inactive FAs unable to flip-flop in a protonated form across the lipid bilayer, but also unable to activate protonophoric function of UCP1 and inhibit its transport of Cl^-. Below, we outline some of these results.

Attempts to indicate the existence of FA binding sites on the mitochondrial integral membrane proteins using radiolabeled palmitate failed (Cunningham *et al*. 1986). Nevertheless, we have provided their indication by the photoaffinity labeling of BAT (Růžička *et al*. 1996) and rat heart mitochondria (Schönfeld *et al*. 1996, Ježek *et al*. 1998, Engstová *et al*. 2001), when [$^3H_4$]-azido-FA did label the most hydrophobic proteins and among them the strongest bands on autoradiograms were represented by the UCP1, ADP/ATP carrier, and phosphate carrier. The isolated UCP1 could bind the photoaffinity label in a quite low stoichiometry, less than 0.5 per dimer (Růžička *et al*. 1996). The best indication of the FA binding site existence in UCP1 was provided by EPR studies using 5-DOXYL-stearic acid (Ježek and Freisleben 1994, Ježek *et al*. 1995), which exhibits a specific EPR signal reflecting the immobilized probe. This specific signal was prevented by lauric acid and alkylsulfonates, and was modulated by the addition of nucleotides. The PN effect on the spin-label signal probably reflects conformational changes involved in the allosteric nucleotide-mediated gating.

Competition between FAs and anionic substrates of UCP1 has also been demonstrated by the kinetic measurements with the reconstituted UCP1 (Ježek *et al*. 1994, 1996a, 1998, Garlid *et al*. 1996, Jabůrek *et al*. 2001), plant UCP (PUMP, Ježek *et al*. 1996b, 1997a, Borecký *et al*. 2001), and recently also with reconstituted UCP2 and UCP3 (Jabůrek *et al*. 1999, Jabůrek and Garlid 2003, Žáčková *et al*. 2003). The "external" translocation sites of UCP1 were found to be shielded, as demonstrated by the failure of non-transported substrate analogs to inhibit transport (Ježek and Garlid 1990). Another support for FA cycling was inferred from the properties of undecanesulfonate uniport via UCP1 (Garlid *et al*. 1996). It competes with FAs for an internal (or hydrophobic binding) site on UCPs but it does not induce H^+ uniport, since it cannot be protonated (only below pH ~2). It can be protonated, however, when the ion-pair with propranolol is formed, which carries H^+ (Jabůrek *et al*. 2001). The third and impressive support for the FA cycling mechanism was provided by revealing the existence and the behavior of so called inactive FAs (Ježek *et al*. 1997b,c). Inactive FAs are unable to flip-flop across the lipid bilayer, as indicated by the lack of flip-flop acidification, i.e. acidification of the vesicle interior upon their addition (Ježek *et al*. 1997b). However, they are also unable to induce H^+ transport with UCP1 and inhibit Cl^- uniport via UCP1 (Ježek *et al*. 1997c). Thus, inhibiting flip-flop, one does not observe any H^+ uniport. Mostly the inactive FAs are the bipolar compounds or exhibit a dumbbell shape. Numerous other inactive FAs were found later (Wojtczak *et al*. 1998, Bernardi *et al*. 2003) and exhibited same properties with the regards to PUMP (Ježek *et al*. 1997a) and UCP2 (Žáčková *et al*. 2003).

Fatty acid cycling mediated by other mitochondrial carriers

Not only UCPs, but several other carriers are considered to conduct FA anions and provide FA cycling. Originally, the FA cycling hypothesis was stated for the ADP/ATP carrier (AAC, Skulachev 1991) as based on the observation of prevention of FA-induced uncoupling by carboxyatractyloside (CAT), a specific AAC inhibitor. Recently, a revolutionary step in bioenergetics was provided by resolving the structure of AAC (Pebay-Peyroula *et al.* 2003). We have demonstrated that mitochondria from yeast lacking AAC do not exhibit such a CAT-sensitivity (Ježek *et al.* 1998), and besides the existence of AAC labeling with azido-FA, we have shown that the observed uncoupling is lost upon covalent attachment of azido-FA (Schönfeld *et al*. 1996). The azido-FA also inhibited ADP uptake. However, note that AAC normally consumes Δp equivalent to pumping of one H^+ – one anionic charge is always expelled, either ATP^{4-} is exchanged for ADP^{3-} when ADP is imported to the matrix, or FA anion is expelled. Since the produced ATP is eventually exported, the energy required for FA anion uniport is dissipating (Ježek *et al* 1998). Also the mitochondrial phosphate carrier (PIC) was shown by us to mediate FA cycling (Žáčková *et al*. 2000, Engstová *et al*. 2001). While studying phosphate analogs which could potentially compete with FAs on PIC, we have revealed the new PIC substrate, methylphosphonate, and novel non-transportable PIC inhibitors, methylenediphosphonate and imino(dimethylenephosphonate) which

also inhibited phosphate transport and FA cycling on PIC.

Ubiquitous UCP2, muscle-specific UCP3 and brain-specific UCP4 and UCP5

Phylogenesis of uncoupling proteins

In the light of the above findings and recognizing that besides 5 UCP genes (Ježek and Urbánková 2000), additional 42 carriers exist in the human genome (Ježek and Ježek 2003), one would conclude that some of these carriers could mediate FA cycling and contribute to the observed leak in mitochondria. The undisputable UCP phylogenesis, beginning from the ancestral prototype UCP of a UCP4 type (Hanák and Ježek 2001), would seem redundant. However, since UCP2 has evolved late in phylogenesis (occurring already in fish or birds) and since UCP3 and UCP1 seem to be the most recent, we must accept that any roles emerged with these proteins were important enough to persist. We do not know why the earliest UCP4 isoform, existing as the sole UCP in *C. elegans* and co-existing with UCP5-isoforms in *Drosophila*, is specific only for the brain of humans, rats, and mice.

Expressed amounts of uncoupling proteins

UCP2 mRNA has been found in all studied human, rat, and mouse tissues (Fleury *et al*. 1997, Gimeno *et al*. 1997) but in different amounts. Due to the revealed translational down-regulation (Pecqueur *et al*. 2001), the actually expressed UCP2 protein amount may differ significantly from the figures given by the popular mRNA quantifications. Thus, the detectable amounts of UCP2 protein were found in lung, spleen, and stomach under normal physiological conditions (Pecqueur *et al*. 2001). This study had to adjust their anti-mouse-UCP2 antibodies to obtain significant differences on wild-type vs. UCP2 (-/-) mice. We have also attempted to indicate UCP2 and UCP3 distribution in various tissues with the own produced low-titer antibodies (Ježek *et al*. 1999). Our polyclonal anti-human-UCP3 antibodies identified UCP2 antigen in rat heart, kidney, brain, and white adipose tissue; and UCP2/UCP3 antigen in rat skeletal muscle. In spite of this, immunological quantifications are difficult and not always convincing (Couplan *et al*. 2002).

Hence, we acquired the completely different strategy based on the quantification of ^{3}H-GTP binding sites in mitochondria (Žáčková *et al*. 2003). From our reconstitution studies we were sure that GDP, ADP, ATP, and GTP inhibit human recombinant UCP2 and UCP3, expressed in yeast (Žáčková and Ježek 2002, Žáčková *et al*. 2003) or *E. coli* (Jabůrek *et al*. 1999, Jabůrek and Garlid 2003). Demonstrating the existence of high affinity ^{3}H-GTP and ^{3}H-ATP binding to recombinant UCP2 (K_d~1.5 μM for yeast expression) and UCP3, expressed either in yeast or *E. coli* (K_ds~5 μM) we obtained a necessary prerequisite for the relevancy of such a method (Žáčková *et al*. 2003). In mitochondria, the saturated ^{3}H-GTP binding revealed the existence of binding sites with even higher affinity (K_ds ~0.2-0.4 μM). Their number was the highest in mitochondria of rat lung (180 pmols per mg protein, Žáčková *et al*. 2003), representing about 22.5 % of the amount of UCP1 in BAT mitochondria. The intermediate number of ^{3}H-GTP binding sites (in pmols per mg protein), 70, was found in rat kidney mitochondria, and quite low numbers, 30 and 20, were found in rat skeletal muscle mitochondria (representing UCP2 plus UCP3 content) and in liver mitochondria, respectively. This pattern fits to the results obtained previously either with mRNA quantification or immunodetection (Pecqueur *et al*. 2001). Moreover, we have found a moderate number of ^{3}H-GTP binding sites (in pmols per mg protein), 91, in rat brain mitochondria (Fig. 1), high number in spleen mitochondria (186, Fig. 1), and 7 times less sites reflecting the plant UCP in mitochondria of maize shoots when compared to those of roots (200, not shown), which again correlated with functional studies (Ježek *et al*. 2000).

Functional activation of UCP2 and UCP3

Human recombinant (yeast-expressed) UCP2 and UCP3 were reconstituted into liposomes and assayed for fatty acid (FA)-induced H^+ efflux (Žáčková and Ježek 2002, Žáčková *et al*. 2003). All natural long chain FAs activated UCP2- or UCP3-mediated H^+ transport. Unlike in report of Echtay *et al*. (2001) we did not observe further significant activating effect by Coenzyme Q_{10}. The absence of this effect was established independently in other laboratory (Jabůrek and Garlid 2003). Evaluated parameters of FA activation (FA cycling) kinetics revealed the highest apparent affinity to UCP2 (the lowest K_ms, 20 μM, 29 μM, respectively) for ω-6 polyunsaturated FAs (PUFAs), all-cis-8,11,14-eicosatrienoic, C20:3(ω-6), and all-cis-6,9,12-octadecatrienoic acids, C18:3(ω-6) (Fig. 3), which are also the most potent agonists of the nuclear PPARβ receptor in the activation of UCP2 transcription

(Chevillotte *et al.* 2001). ω-3 PUFAs, cis-5,8,11,14,17-eicosapentaenoic acid, C20:5(ω-3), and cis-4,7,10,13, 16,19-docosahexaenoic acid, C22:6(ω-3), had lower affinities (K_m 50 μM and ~60 μM, respectively). Although being ω-6 PUFA, arachidonic acid exhibited the same low affinity (K_m 250 μM) as lauric and palmitic acid (K_m for both ~200 μM). Surprisingly, low was affinity for oleic acid (K_m 334 μM) and myristic acid (K_m 450 μM), which however exhibited quite high V_{max}, higher than those roughly estimated for PUFAs (Žáčková *et al*. 2003). UCP2- (UCP3)-dependent H^+ translocation activated by all tested FAs was inhibited by purine nucleotides with apparent affinity to UCP2 (reciprocal K_i) decreasing in order: ADP > ATP ~ GTP > GDP >> AMP. All these findings shows that the phenotype of UCP2 and UCP3 is similar to the UCP1-phenotype, i.e. FA-activated H^+ uniport, inhibited by PN. UCP2 (UCP3) can be activated in the presence of FAs and upon simultaneous release of PN from their binding site. This can be achieved by modulators of PN inhibitors. Among them Mg^{2+} and alkaline pH diminish PN binding to UCP1 (Ježek *et al*. 1988) (effects on UCP2 PN binding are not known). A modulator can be any compound competing with PN but not inhibiting UCPs.

The special place among FAs is given to PUFAs. Novelty of our findings with PUFAs lies in suggestion of a possible dual role of some PUFAs in activating both UCP2 expression and uncoupling activity. Physiological meaning of such activation is even more significant. PUFAs, their hydroperoxides, and hydroxyl FA derivatives originating from lipoperoxidation, can be cleaved off by phospholipase A and may activate UCP2 instantly. As the following chapter describes, such activation represents a feedback down-regulation of ROS formation. In order to judge the physiological significance of UCP2 activation by PUFAs, one needs to consider the PUFA content *in vivo*. Surprisingly, it is not as low as expected. C20:3(ω-6) and C18:3(ω -6) content in hepatic arterial plasma phospholipids accounts for 2.7 % and 0.2 %, respectively; while C20:5(ω-3) and C22:6(ω-3) for 1.3 % and 3.7 %, respectively (Clemmesen *et al*. 2000). Content of C20:3(ω-6) and C18:3(ω-6) in total lipids extracted from rat liver was 1.9 and 0.8 ng/μg lipids (2.9 and 0.4 in kidney), respectively (Deiana *et al*. 2001). There is 103 ng/μg lipids of arachidonic acid (AA) in liver and 84 ng/μg lipids in kidney; C20:5(ω-3) and C22:6(ω-3) were 1.7 and 39 ng/μg lipids in liver and 1.7 and 10 ng/μg lipids in kidney. Total concentrations found in typical human plasma samples in μg/ml were found as follows: 48 for 20:3(ω-6); 11 for C18:3(ω-6), 23 for C20:5(ω-3), and 55 for C22:6(ω-3), in contrast to 228 for AA and 1221 for linoleic acid (Bailey *et al*. 1998). The content of C20:3(ω-6) in phospholipids slightly increases in maternal plasma before delivery: accounting for changes between 3 to 3.5 μg/ml, whereas values of ω-3 PUFAs are slightly decreasing (values in μg/ml) as from 0.55 to 0.35 for C20:5(ω-3), from 4 to 3.87 for C22:6(ω-3) (Rump *et al*. 2001). Elongation of C18:3(ω-6) to C20:3(ω-6) may occur in some cell types more rapidly than does desaturation to AA. This leads to higher C20:3(ω-6) to AA ratios within certain cells after supplementation of C18:3(ω-6) (Johnson *et al*. 1997). Serum C20:3(ω-6) was thus elevated from 100 μM in serum lipids up to 300 μM (whereas AA from 600 μM up to 850 μM). One can speculate that if 10 % of the above mentioned PUFA amounts would be cleaved off, a substantial UCP2 activation will occur. Also due to the high C22:6 ω-3 content in the brain or retina tissues, activation of UCP2, (UCP4, UCP5) by C22:6 ω-3 is very plausible.

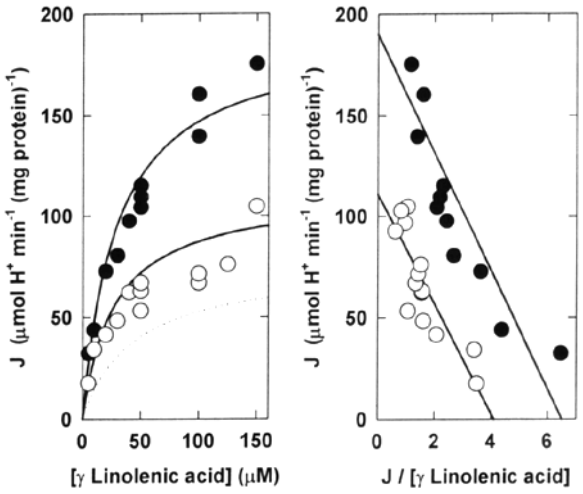

Fig. 3. Activation of reconstituted human recombinant (yeast expressed) UCP2 by γ-linolenic acid. Left panel show the direct plots of γ-linolenic-acid-induced H^+ flux with increasing total γ-linolenic acid concentration for the absence (filled symbols) and presence (open symbols) of 2.5 mM ADP; while the right panel illustrates the Eadie-Hofstee plot for the same data. The dotted line represents theoretical fits by the Michaelis Menten-equation for the differential H^+ fluxes, when fluxes in the presence of ADP were subtracted from the control ones. The derived K_m corresponded to 29 μM and V_{max} to 190 μmol $H^+.min^{-1}$ .mg $protein^{-1}$.

UCPs and down-regulation of reactive oxygen species (ROS) production

Reactive oxygen species production in mitochondria

About 1-2 % of oxygen is converted to superoxide anion ($O_2^{-\bullet}$) in mitochondria at Complex I (at sites generating semiquinones) and Complex III (on the site proximal to matrix on which regeneration of oxidized coenzyme Q, ubiquinone, UQ, to its reduced form UQH_2 proceeds via ubisemiquinone anion radical ($UQ^{-\bullet}$) (Raha and Robinson 2000, Pedersen 1999, Skulachev 1998). Superoxide half-life is <1 μs, since an array of radical and non-radical compounds is produced which are called reactive oxygen species (ROS). ROS include radicals $O_2^{-\bullet}$, $OH^{\bullet}$, peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), as well as nonradical compounds such as H_2O_2 or HOCl, and a subgroup of reactive nitrogen species (RNS), such as nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^{-\bullet}$). Although a minor portion of $O_2^{-\bullet}$ (1 % at pH 6.8) is hydrated to $HO_2^{\bullet}$ (the third most reactive radical after $RO_2^{\cdot}$ and $OH^{\cdot}$), the majority of $O_2^{-\bullet}$ is converted to H_2O_2 by matrix Mn-superoxide dismutase (MnSOD) and intramembrane space or cytosolic CuZnSOD; H_2O_2 is then processed by glutathione peroxidase but may also be converted into $OH^{\bullet}$ by reactions catalyzed by metals, namely by Fe-catalyzed Fenton reaction. $HO_2^{\bullet}$ and $OH^{\bullet}$ initiate substantial lipoperoxidation which spreads radical propagation. It produces hydroperoxy-FA radicals of unsaturated hydrocarbon chains, which are cleaved off by phospholipase A_2 in the presence of Ca^{2+}. The resulted FA hydroperoxides are transient non-radical but reactive species, which are degraded by glutathione peroxidase. Before this, they could activate UCP2 similarly as do the maternal PUFAs (Žáčková *et al*. 2003).

Uncoupling decreases the reactive oxygen species production in mitochondria

Any slight increase of the H^+ backflux (to the matrix), which diminishes Δp, results in a substantial decrease of mitochondrial ROS formation (Korshunov *et al*. 1997). It can be explained on the basis of increased respiration due to the respiratory control. Slightly increased respiration shortens lifetime of ubisemiquinone anion radical ($UQ^{-\bullet}$) and leads to lowered oxygen tension in the microenvironment. Both processes cause reduced rate of $O_2^{-\bullet}$ formation. Naturally such an increased H^+ backflow proceeds via the F_O part of ATP-synthase. Hence also during the transition between State 4 (non-phosporylating resting respiration) to State 3 (ATP synthesis) ROS formation is drastically reduced. By other words, most of ROS are produced *in vivo* under the non-phosphorylating "resting" state. The H^+ backflow given by uncoupling (of any type, by leak or protein-mediated uncoupling) also decreases accordingly the rate of ROS formation rate. This phenomenon in heart mitochondria was quite impressive: ΔΨ decrease by 10 % led to 55 % decrease in ROS production (monitored as H_2O_2 production, Korshunov *et al*. 1997). Consequently, even the mild uncoupling given by UCP2 (or UCP3 to UCP5), when activated by FAs and by releasing PN inhibition, can intensively down-regulate ROS production (Nègre-Salvayre *et al*. 1997, Arsenijevic *et al*. 2000).

Uncoupling protein UCP2 is part of a feedback loop leading to accelerated down-regulation of ROS production in mitochondria

Downregulation of mitochondrial ROS production seems to be the most plausible role for UCP2, since its expression is expected in numerous mammalian tissues, yet in minute amounts. The same considerations are valid for UCP3 in skeletal muscle and UCP4 and UCP5 in the brain. Thus the report of Nègre-Salvayre *et al*. (1997) could already be interpreted as suppression of ROS production due to the UCP2 function. They observed an increased H_2O_2 production due to ΔΨ increase induced by GDP addition, likely mediated by UCP2 in macrophage (liver Kupffer cell) mitochondria or in thymus and spleen mitochondria. In leptin-deficient *ob*/*ob* mice, low UCP2 levels in macrophages are found together with the increased macrophage mitochondrial ROS production when compared to normal mice (Lee *et al*. 1999). Also, the UCP2(-/-) (Arsenijevic *et al*. 2000) and UCP3(-/-) mice (Vidal-Puig *et al*. 2000) exhibited higher levels of ROS in macrophages and muscle, respectively. Moreover, Brand's group (Echtay *et al*. 2002a) promoted the idea that superoxide itself activates UCP2 by unspecified mechanism from the matrix side (Echtay *et al*. 2002b). Recently, they rather ascribed such ability to the end-product of the lipoperoxidation cascade for ω-6 PUFAs, to 4-hydroxy-2-nonenal (Echtay *et al*. 2003). In addition, Skulachev and Goglia (2003) speculated that FA hydroperoxides can be anionic transport substrates of UCPs, but predicted that they cannot diffuse through the membrane in a protonated form. However, our own measurements indicate that the second aspect is not true (Jabůrek *et al*., unpublished data) and that FA

hydroperoxide can undergo the cycling mechanism enabled by UCPs similarly as regular FAs. Activation of UCP2-mediated uncoupling by FA hydroperoxides could provide a feedback control mechanism by which increasing FA hydroperoxides, as the lipoperoxidation product and hence also the product of the increased ROS production, activate UCP2-mediated uncoupling, which in turn leads to the suppression of ROS production. This activation persists longer than for a period in which all hydroperoxides are degraded by the glutathione peroxidase, since the resulting hydroxy FAs can also activate UCPs. Similarly, the activation of UCP2 by superoxide promoted by Brand's group (Echtay *et al*. 2002a,b) could be in fact activation by lipoperoxidation products such as FA hydroperoxides and hydroxy FAs, formed downstream in the ROS propagation cascade.

Mitochondrial suppression of ROS production could exhaust ROS from intracellular and extracellular space

The ability to reduce ROS not only in own mitochondria, but within the cell or even in the extracellular space has also been ascribed to UCP2. Duval *et al*. (2002) have recently shown that UCP2-mediated uncoupling in endothelial cells is even able to decrease extracellular ROS in coincubated low-density-lipoproteins (LDL). Furthermore, mice with deleted LDL receptor exhibited extensive diet-induced atherosclerotic plaques when they received bone marrow transplanted from UCP2 (-/-) mice, and appearance of these plaques was prevented when they received bone marrow transplants from UCP2 (+/+) mice (Blanc *et al*. 2003). These findings suggest a hypothesis in which ROS or redox homeostasis in the whole tissue or even of the whole organism is regulated by ROS-sink in mitochondria where UCP2 (or UCPn)-mediated suppression of ROS production takes place. Hence, the basic role of all UCPs could be to avoid oxidative injury to organelles and tissues. Balanced regulations of UCPn may contribute to prolonged lifetime of organism.

Perspectives of uncoupling protein research

Participation of UCP2 in prevention of atherosclerosis and in signal transduction involving ROS are the two future directions implied in the previous chapter. However, other aspects not covered in this review, will put UCP research onto the "cutting edge" – revealing details of UCP2 participation in certain etiologies of type 2 diabetes, understanding whether UCPn-induced uncoupling participates in apoptotic signaling, and, last but not least, definitive confirmation whether UCP2 and UCP3 participate in fever and other types of adaptive thermogenesis and whether dysfunctions in UCPs or their regulations lead to obesity.

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