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REVIEW

Antioxidant and Regulatory Role of Mitochondrial Uncoupling Protein UCP2 in Pancreatic β-cells

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Summary

Research on brown adipose tissue and its hallmark protein, mitochondrial uncoupling protein UCP1, has been conducted for half a century and has been traditionally studied in the Institute of Physiology (AS CR, Prague), likewise UCP2 residing in multiple tissues for the last two decades. Our group has significantly contributed to the elucidation of UCP uncoupling mechanism, fully dependent on free fatty acids (FFAs) within the inner mitochondrial membrane. Now we review UCP2 physiological roles emphasizing its roles in pancreatic β-cells, such as antioxidant role, possible tuning of redox homeostasis (consequently UCP2 participation in redox regulations), and fine regulation of glucose-stimulated insulin secretion (GSIS). For example, NADPH has been firmly established as being a modulator of GSIS and since UCP2 may influence redox homeostasis, it likely affects NADPH levels. We also point out the role of phospholipase iPLA2 isoform γ in providing FFAs for the UCP2 antioxidant function. Such initiation of mild uncoupling hypothetically precedes lipotoxicity in pancreatic β-cells until it reaches the pathological threshold, after which the antioxidant role of UCP2 can be no more cell-protective, for example due to oxidative stress-accumulated mutations in mtDNA. These mechanisms, together with impaired autocrine insulin function belong to important causes of Type 2 diabetes etiology.

Key words

Mitochondrial uncoupling protein UCP2 • Pancreatic β-cells • Homeostasis of reactive oxygen species • Redox regulations • Mitochondria • Glucose-stimulated insulin secretion

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Introduction

Research on brown adipose tissue, brown adipose tissue mitochondria and later, after its discovery, research on the mitochondrial uncoupling protein UCP1, has been conducted for half a century and has been traditionally studied in the Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic (Novák *et al.* 1965, Drahota *et al.* 1968, 1970, Hahn 1970, Houštěk and Drahota 1975, 1977, Houštěk *et al.* 1978, Svoboda *et al.* 1981, Kopecký *et al.* 1984, 1987, Ježek *et al.* 1988, 1989, 1990a,b, Ježek and Drahota 1989). Our group has significantly contributed to the elucidation of UCP uncoupling mechanism, fully dependent on free fatty acids (FFAs) within the inner mitochondrial membrane (see Chapter 2.1). In 1997, UCP2 with widespread distribution in tissues has been discovered (Gimeno *et al.* 1997, Fleury *et al.* 1997) and since then the idea started that physiological uncoupling should serve to important function. That is why we review UCP2 physiological roles emphasizing its roles in pancreatic β-cells, such as antioxidant role, possible tuning of redox homeostasis (consequently UCP2 participation in redox regulations), and fine regulation of

glucose-stimulated insulin secretion (GSIS).

Delicate redox homeostasis in pancreatic β-cells

Mitochondrial reactive oxygen species (ROS) sources and redox buffers

Similarly to other cell types (Ježek and Hlavatá 2005), mitochondrial respiratory chain is the main source of superoxide ($O_2^{\bullet-}$, and its conjugated acid-hydroperoxyl radical, $HO_2^{\bullet}$, pKa 4.9) in mitochondrion of pancreatic β-cells. Complex I, an H^+-pumping NADH:quinone oxidoreductase, is considered to produce maximum superoxide only when both electron transport and H^+ pumping are retarded (Dlasková *et al*. 2008a,b). H^+ pumping may be attenuated by high electrochemical gradient of protons established at inner mitochondrial membrane (IMM), termed proton-motive force, Δp, when expressed in mV units; or inhibited by oxidative stress-related mutations of ND5 subunit (or other mitochondrion-coded subunits). Intermediate $O_2^{\bullet-}$ formation results from fully reduced flavin as reported for isolated Complex I (Pryde and Hirst 2011). Binding of rotenone and similar inhibitors in proximity to the Q-site (a ubiquinone binding site) highly retards electron transport throughout the peripheral arm of Complex I. This was originally ascribed to the formation of longer-lived semiquinone species having a higher probability of reacting with oxygen which thus would form $O_2^{\bullet-}$ (Brand *et al*. 2004). Nevertheless, a detailed mechanism of $O_2^{\bullet-}$ formation within Complex I and its relation to H^+-pumping have yet to be established. It is well recognized, however, that nearly all Complex I-produced $O_2^{\bullet-}$ is released to the matrix compartment (Brand *et al*. 2004). Complex III, a ubiquinol-cytochrome c reductase, contributes to $O_2^{\bullet-}$ generation by autooxidation of the ubisemiquinone anion radical ($UQ^{\bullet-}$) within so-called Q cycle (Muller *et al*. 2003, Brand *et al*. 2004, Ježek and Hlavatá 2005), while it releases $O_2^{\bullet-}$ about equally to both sides of the inner mitochondrial membrane (IMM, Muller *et al*. 2003, 2004).

A fast electron flux *via* the whole respiratory chain at a high substrate pressure ($NADH/NAD^+$ ratio) produces more $O_2^{\bullet-}$ than under conditions, when slower flux occurs at the same relative retardation (same oxidation/reduction states). Hence, in intact respiratory chain, mostly effectors that retard cytochrome c turnover between Complex III and IV (cytochrome c oxidase), slow down Q cycle or CoQ migration between Complex I and III, accelerate superoxide production (Ježek and Plecitá-Hlavatá 2009).

As we discussed earlier, mitochondria represent an important cellular ROS source, which may be under certain circumstances dominant (Ježek and Hlavatá 2005). Non-mitochondrial ROS sources and their significance for pancreatic β-cells have been reviewed recently by us (Ježek *et al*. 2012) as together with cell antioxidant and redox buffer systems determine the overall ROS homeostasis and hence also the development of oxidative stress (Ježek and Hlavatá 2005). In pancreatic β-cells a strong non-mitochondrial ROS source is represented by NADPH oxidases, namely isoforms NOX1, 2, and 4 (Newsholme *et al*. 2009). Together with the weak antioxidant defense system and low capacity of redox buffers in pancreatic β-cells, it creates a delicate ROS homeostasis, which might be disturbed by a rather weak insult (Lenzen *et al*. 1996, Tiedge *et al*. 1997).

Likewise in all cell types, redox buffers and antioxidant enzymes in pancreatic β-cells are contained in mitochondrial matrix, cytosolic and other cell compartments (organelles, Ježek *et al*. 2012). Redox buffers and antioxidant enzymes detoxify the produced ROS and may exert specific roles in redox signaling. Catalase (absent in mitochondria except of the heart), glutathione peroxidase (GPX), and superoxide dismutase (SOD1 or CuZnSOD) represent the three of the most important intracellular antioxidant enzymes, a primary defense system. Whereas SOD2 or MnSOD and GPX4 are specific for mitochondrial matrix, SOD1 also localizes to the mitochondrial intermembrane space. However, the expression and activity of antioxidant enzymes is low in rodent β-cells compared to other organs (Lenzen 2008). This property increases their susceptibility to oxidative insult. Besides vitamin E (α-tocopherol), ascorbate and uric acid, among small antioxidant molecules, glutathione provides an important mechanism protecting β-cells against oxidative damage (Krause *et al*. 2011). Glutathione, present in mM concentrations, is kept in the reduced state (GSH) by glutathione reductase. GSH transfers its reducing equivalents to ascorbate, GPX, and glutaredoxins.

The main protein antioxidant defense is composed of disulfide reductases, namely thioredoxin (TRX), glutaredoxin (GRX), peroxiredoxins (PRX) and glutamate-cysteine ligase. Thioredoxin represents a disulfide reductase for protein sulfhydryl groups, maintaining proteins in the reduced state (Bachnoff *et al*. 2011). Thioredoxin reductase uses electrons from

NADPH and regenerates oxidized TRX. Similarly, glutaredoxin reductase-2 (Reinbothe *et al*. 2009) reduces H_2O_2 or hydroperoxy-fatty acyl lipid chains to water or hydroxy lipid chains, respectively, at the expense of conversion of GSH to oxidized glutathione GSSG, which is regenerated by glutathione reductase. Peroxiredoxins are a family of thiol peroxide reductases which uses TRX or other thiol-containing proteins to clear H_2O_2 or lipid peroxides (Zhao and Wang 2012). Peroxiredoxin reaction product is sulfenic acid. At the TRX shortage, peroxiredoxin is inactivated to PRX-SO_2 (Yang *et al*. 2002), which can be reversed by sulfiredoxins, at the expense of ATP, yielding PRX-SOH.

Mild uncoupling attenuates mitochondrial ROS generation at intact mtDNA

Oxidative phosphorylation (OXPHOS) at mitochondrial ATP synthase (Complex V) is driven by the protonmotive force, Δp, formed by the respiratory pumping chain H^+ at Complex I, III, and IV. The IMM domain of ATP synthase, F_OATPase, consumes an adequate Δp portion in a state, historically termed state-3. *In vivo* cellular respiration is governed by the metabolic state and/or availability of substrates, a finely tuned spectrum of various states-3 can be established, depending on the substrate load (e.g. increasing glucose). A state-4, is then given by zero ATP synthesis, when zero H^+ backflux *via* the F_OATPase proceeds while respiration and H^+ pumping are given by so-called H^+ leak, mediated by mitochondrial carrier proteins as their side-function or given by the native H^+ permeability of IMM. Since mitochondrial Δp is predominantly in the form of $\Delta\Psi_m$, IMM electrical potential, $\Delta\Psi_m$ is maximum at state-4 at the maximum substrate load. Besides other proteins, such as the ADP/ATP carrier, dissipation of the protonmotive force within IMM, a protonophoric short-circuit, also known as uncoupling, can be physiologically provided by mitochondrial uncoupling proteins (UCPs) (see below). When carrier-mediated protonophore activity plus IMM H^+ leak does not overwhelm the F_OATPase protonophoric activity, then ATP synthesis, hence OXPHOS, still takes place. Such a mild uncoupling (mild in contrast to a complete uncoupling by agents termed uncouplers) is, however, beneficial in terms of lowering mitochondrial $O_2^{\bullet-}$ formation. $O_2^{\bullet-}$ formation at both Complex I (Dlasková *et al*. 2008a,b) and Complex III (Korshunov *et al*. 1997) was reported to be diminished by mild uncoupling. Due to a relative predominance of mitochondrial ROS source within the cell, one can predict that even accumulated oxidative stress might be attenuated by mild uncoupling. Note, however, that oxidative stress originating from irreversible changes, such as stress due to mutated subunits encoded by mitochondrial DNA (mtDNA) cannot be improved by mild uncoupling (Dlasková *et al*. 2008a). An example is given by certain mutations of ND5 subunit of Complex I (ensuring H^+ pumping in intact wild-type form) that inhibit H^+ pumping and lead to increased $O_2^{\bullet-}$ formation. Such a block is not withdrawn by uncoupling. In conclusion, retardation of H^+ pumping which accelerates Complex I $O_2^{\bullet-}$ formation rather initiates further turn of a vicious spiral of self-accelerated oxidative stress (Dlasková *et al.* 2008a).

Oxidative phosphorylation (OXPHOS) as determinant of glucose-stimulated insulin secretion (GSIS) but also mitochondrial ROS generation

Pancreatic β-cells sense glucose *via* elevated OXPHOS (Ashcroft and Rorsman 2012). Their respiration and OXPHOS rates, leading to a certain ATP/ADP ratio, are strictly given by the availability of glucose, whereas in most other cell types it is the other way around – cell demand dictates respiration/ metabolism rates and the ATP/ADP ratio. It is because pyruvate cannot be easily diverted towards lactate dehydrogenase for lactate formation and therefore β-cells cannot metabolize glucose by aerobic glycolysis. Canonical mechanism has been established predicting that the increased ATP/ADP ratio in β-cell cytosol initiates more frequent closure of the ATP-sensitive K^+-channels (Bennet *et al*. 2010, Szollosi *et al*. 2010, Soty *et al*. 2011), thus depolarizing plasma membrane and activating voltage-gated L-type Ca^{2+}-channels (Rorsman *et al*. 2012). The resulting Ca^{2+} entry elevates submembrane Ca^{2+} concentration and stimulates Ca^{2+}-dependent exocytosis of insulin-containing secretory granules (Ashcroft and Rorsman 2012).

For cells not completely depleted of glucose, we hypothesized (Ježek *et al*. 2012) that the release of superoxide to the mitochondrial matrix upon the GSIS onset is diminished with regard to the release rates at lower glucose concentrations. GSIS should simultaneously result in the decrease of mitochondrial oxidative stress. The incremental increase of electron flow through the respiratory chain is not high at ~3mM glucose, and its rise due to a further glucose intake is relatively lower when compared with the effect of H^+ backflow *via* the F_O part of ATP synthase that elevates

respiration (classic respiratory control for isolated mitochondria). Thus the effect of elevated OXPHOS intensity prevails and ROS production is attenuated. This should be valid also for decrease of mitochondrial ROS formation with decreasing ADP, hence increasing ATP (Fridlyand and Philipson 2004) and has been experimentally observed (Koshkin *et al*. 2003). In turn, at extensive glucose depletion, the effect of substrate load (a directly proportional increase in superoxide formation, e.g. on Complex I, with increasing NADH or respiration) should overcome the suppressing role of H^+ returning *via* F_OATPase at higher intensity of OXPHOS. Hence, experimentally, results of increasing mitochondrial ROS upon GSIS might be observed using dihydrodichloro-fluorescein diacetate fluorescent probe (CM-H2DCFDA, further abbreviated DCF) (Bindokas *et al*. 2003, Sakai *et al*. 2003, Leloup *et al*. 2009) as well as increasing reducing equivalents (Patterson *et al*. 2000).

Since H_2O_2 of mitochondrial origin may readily access cytosol, one may report on mitochondrial ROS contribution, when measuring cytosolic ROS sensitive to mitochondrial inhibitors. As explained above, a various extent of glucose depletion may provide distinct outcome in ROS assays, which are further dependent on the employed probe. Thus using dihydroethidium fluorescent monitoring in primary rat β-cells, Martens *et al*. (2005) have found that unlike in non-β-cells, oxidative stress diminishes with increasing glucose upon GSIS. ROS decrease monitored by DCF upon GSIS has also been indicated in isolated Langerhans islets (Lacraz *et al*. 2009). Other laboratories have reported increases in ROS upon GSIS (Bindokas *et al*. 2003, Sakai *et al*. 2003, Leloup *et al*. 2009). Note, that insulin secretion in INS1 cells was also induced by exogenous H_2O_2 and diethyl maleate (Pi *et al*. 2007), or by mono-oleoyl-glycerol (Saadeh *et al*. 2012), which increase intracellular H_2O_2.

Autocrine insulin and mitochondrial ROS generation

Autocrine insulin has acute (4 h) effects on GSIS in healthy humans (Bouche *et al*. 2010). Studies of Poderoso group have pointed out an emerging role of mitochondrial NO synthase (mtNOS) activated upon insulin signaling *via* the Akt-2/protein-kinase-B-mediated phosphorylation in skeletal muscle (Finocchietto *et al*. 2008). Released nitric oxide, a freely permeable radical, $NO^\bullet$, having a half-life of 1 to 10 s, causes a mild oxidative and nitrosative stress but also transiently diminishes respiration. In skeletal muscle and liver $NO^\bullet$ facilitates conversion of glucose to glycogen. Experimentally, it has been demonstrated by a sustained insulin dosage that the insulin-Akt-2-mtNOS pathway mediates $NO^\bullet$ burst in skeletal muscle (Finocchietto *et al*. 2008). Also, nitric oxide donors increase glucose uptake in primary human skeletal muscle cells (Henstridge *et al*. 2009). Signaling *via* phosphatidyl-inositol-3-kinase (PI3K) (and hence downstream Akt-2 signaling) was responsible for insulin receptor activation by nonpeptidyl mimetic L-783,281 which inhibited GSIS as well as basal insulin secretion in human islets of Langerhans (Persaud *et al*. 2002). Also a direct observation in isolated mitochondria that insulin signaling regulates mitochondrial function in β-cells has been reported (Liu *et al*. 2009).

Since pancreatic β-cells contain a functional insulin receptor (Kulkarni *et al*. 1999, Brennand *et al*. 2007, Okada *et al*. 2007), an acute autocrine insulin signaling may lead to similar acute effects as in skeletal muscle and liver, besides chronic positive effects on stimulation of β-cell proliferation (Brennand *et al*. 2007), hence being beneficial for regulation of adult β-cell mass. Transgenic mice lacking insulin receptor in pancreatic β-cells (βIRKO mice) exhibited increased apoptosis, decreased proliferation, and reduced β-cell mass (Kulkarni *et al*. 1999). The insulin receptor has also been found essential for islet compensatory growth response to insulin resistance (Okada *et al*. 2007). There are two arms of autocrine insulin signaling *via* insulin receptor, the Raf-1 kinase arm and the Akt kinase arm. Insulin stimulates primary β-cell proliferation *via* Raf-1 kinase and suppresses apoptosis. The Akt arm increases β-cell mass and improves glucose tolerance. A signalosome complex of glucokinase, pro-apoptotic protein, Bcl-2-associated death promoter, BAD_S, and protein kinase A has been reduced in βIRKO mice, thus linking a lack of autocrine insulin with development of Type 2 diabetes (Liu *et al*. 2009).

If mtNOS is indeed activated upon insulin signaling in β-cells, the predicted outcome may substantiate different roles than in skeletal muscle cells and hepatocytes, just due to the impossibility to switch to a partial aerobic glycolysis and provide a spectrum of anaplerotic pathways. The released $NO^\bullet$ may transiently inhibit Complex I and cytochrome c oxidase. $NO^\bullet$ may also react with superoxide, thus forming peroxynitrite which can further act against otherwise diminishing mitochondrial superoxide production.

Mitochondrial uncoupling proteins

Decades of uncoupling protein research

Mitochondrial uncoupling proteins (UCPs) belong to the SLC25 anion carrier family, having 46 members, among which five UCP isoforms have been identified (Palmieri 2013). The historically first, UCP1, has been discovered and ascribed as specific to brown adipose tissue (BAT) where it provides the final key unit of catabolic cascade of nonshivering thermogenesis (Cannon *et al*. 2006). Nevertheless, our present knowledge indicates that thermogenesis is given not only by UCP1 expression but also by specific composition of BAT mitochondria, namely the lowered ATP synthase content. All other UCPs due to the lack of such a specific mitochondrial set-up and due to much lower amounts existing in tissues do not provide excessive heat release and rather tune OXPHOS efficiency in a way to regulate complex molecular physiology of mitochondria within the cell. It is difficult to assess the physiological impact of UCP2-mediated uncoupling of OXPHOS because the minute amounts of UCP2 expressed in tissues give rise to a small effect, leading only to a small decrease in IMM potential ($\Delta\Psi_m$) on the order of single-millivolts that are difficult to measure (Ježek *et al*. 2004).

UCP2, as a second discovered isoform by Tartaglia (Gimeno *et al*. 1997) and independently reported by Ricquier's and Warden's group (Fleury *et al*. 1997), was first characterized *via* its transcript widely distributed in all mammalian tissues, whereas UCP3 transcript was found specifically in BAT, skeletal muscle and heart (Boss *et al*. 1997, Vidal-Puig *et al*. 1997). Apparently more brain-specific isoforms UCP4 (Mao *et al*. 1999) and UCP5 (originally called BMCP) have been also identified (Sanchis *et al*. 1998, Yu *et al*. 2000, Kim-Han *et al*. 2001, Lengacher *et al*. 2004).

The two aspects now revealed had delayed understanding of UCP functions and physiological roles. At first, translational downregulation (Pecqueur *et al*. 2001, Hurtaud *et al*. 2006) diminishing UCP expression, likewise up-regulation has been described (Hurtaud *et al.* 2007), so the protein amount is not proportional to the transcript. Moreover, UCP2 lifetime has been found to be extremely short, so regulations of its expression possess a nearly direct switch-on/of regulation of UCP2 presence. Simultaneously, as predominant integral membrane proteins with dimeric six membrane-spanning α helices, UCPs are difficult to be selectively recognized by antibodies and cross-reactions occurs with all 46 SLC25 family members (Ježek *et al*. 1999, Pecqueur *et al.* 2001). The second aspect was lying in the disputes on the own uncoupling mechanism as described below, and led to theoretical misconceptions such as consideration of fatty acid (FA) export by UCP3 (Seifert *et al*. 2008).

From the bioinformatics point of view, uncoupling proteins UCP1, UCP2, UCP3, UCP4, and UCP5 form a distinct subfamily within the gene family of mitochondrial anion carriers (Ježek and Urbánková 2000, Hanák and Ježek 2001, Ježek and Ježek 2003, Klingenspor *et al*. 2008). In terms of homology, the closest carrier to UCPs is the oxoglutarate carrier, which, however, lacks the unique uncoupling protein signature sequences (Ježek and Urbánková 2000, Hanák and Ježek 2001, Ježek and Ježek 2003).

The advent of gene ablation and silencing led to influential pioneer findings showing surprising UCP roles. Thus already the report of Nègre-Salvayre *et al*. (1997) could be interpreted as suppression of ROS production due to the UCP2 function. They observed an increased H_2O_2 production due to $\Delta\Psi_m$ increase induced by GDP addition, likely mediated by UCP2 in macrophage (liver Kupffer cell) mitochondria or in thymus and spleen mitochondria. UCP2(-/-) mice were more resistant to *Toxoplasma gondii* infection due to higher macrophage attack (Arsenijevic *et al*. 2000), excellently demonstrating how mitochondrial ROS homeostasis affects not only cellular but also extracellular ROS homeostasis (detailed description in Ježek and Hlavatá 2005). Simply, higher mitochondrial ROS production due to the lack of UCP2 antioxidant function had spread towards cytosol, overwhelmed the redox buffers and antioxidant mechanisms therein, hence more ROS were left for microbe killing. Actually, the additional ROS were probably superimposed to the classic macrophage activated NADPH oxidase ROS formation. Similarly, UCP3(-/-) mice exhibited higher levels of ROS in muscle (Vidal-Puig *et al*. 2000). Another surprise came from the suggestion that UCP2 regulates glucose-stimulated insulin secretion (GSIS, Zhang *et al*. 2001, Krauss *et al*. 2005, Parker *et al*. 2009, see below). But this is just one of numerous examples how fine tuning of OXPHOS may be related to crucial physiological phenomena. Another such example (Trenker *et al*. 2007, Wu *et al*. 2009) is the observation that UCP2 may be fundamental for the regulation of Ca^{2+} levels in mitochondria. Indeed, uncoupling is strictly affecting Ca^{2+} uptake and efflux *via* IMM, diminishing $\Delta\Psi_m$-dependent Ca^{2+} uniport uptake, likewise

ΔpH-dependent Ca^{2+} efflux *via* Ca^{2+}/Na^{+} and Ca^{2+}/H^{+} carriers. Even though such a physiological role for UCP2 has been challenged by other groups (Brookes *et al*. 2008), we may speculate that the observation of UCP2-dependent Ca^{2+} uptake may reflect the ability of Ca^{2+}-complexed fatty acid anions to interact with UCPs in combination of Δp effects on Ca^{2+} fluxes.

A number of studies also pointed to the attention to UCP2 in cancer cells (Baffy 2010). UCP2 overexpression has been reported for a variety of cancer cells and linked to enhanced tumor formation in the soft agar or xenograft model (Ayyasamy *et al*. 2011). Several lines of evidence also suggested a role of UCP2 in cancer chemoresistence. For instance, UCP2 overexpression in colon cancer cells resulted in diminished apoptosis (caspase-3 activation) in response to etoposide, doxorubicine, CPT and UV radiation *in vitro* as well as in xenograft studies with UCP2 overexpressing cells, by suppressing the phosphorylation of p53 within the transactivating domain *via* inhibition the ROS production (Derdak *et al*. 2008). UCP2 upregulation should help cells to escape from apoptosis mediated by the p53 signaling. UCP2-dependent chemoresistance is believed to be based on quenching of drug-induced ROS burst by promoting proton leak. Indeed, inhibiting of UCP2 by diamine-induced glutathionylation (Pfefferle *et al*. 2013) or siRNA (Dalla Pozza *et el*. 2012) causes augmented drug sensitivity using various chemotherapeutics.

Recently, UCP1 has also been detected in thymocytes (Carroll *et al*. 2005, Adams *et al*. 2008a,b), where its thermogenic role is probably replaced by a regulatory role in apoptosis due to its ability to attenuate mitochondrial ROS production (Dlasková *et al*. 2006, 2010).

Uncoupling mechanism of mitochondrial uncoupling proteins – fatty acid wobbling

In spite of the fact that the crystallographic structure of UCP2 has been described (Berardi *et al.* 2011), likewise the structure of the prototypical SLC25 family member, the ADP/ATP carrier (Pebay-Peyroula *et al*. 2003), molecular mechanism of uncoupling was not deduced from structure but from numerous functional studies of reconstituted UCPs. We have been for long time involved in this research (e.g. Ježek *et al*. 1997a,b, Urbánková *et al*. 2003, Žáčková *et al*. 2003, Jabůrek *et al*. 2004) and such pioneer studies turned out to be closest to the recently established model (Fedorenko *et al*. 2012) (Fig. 1).

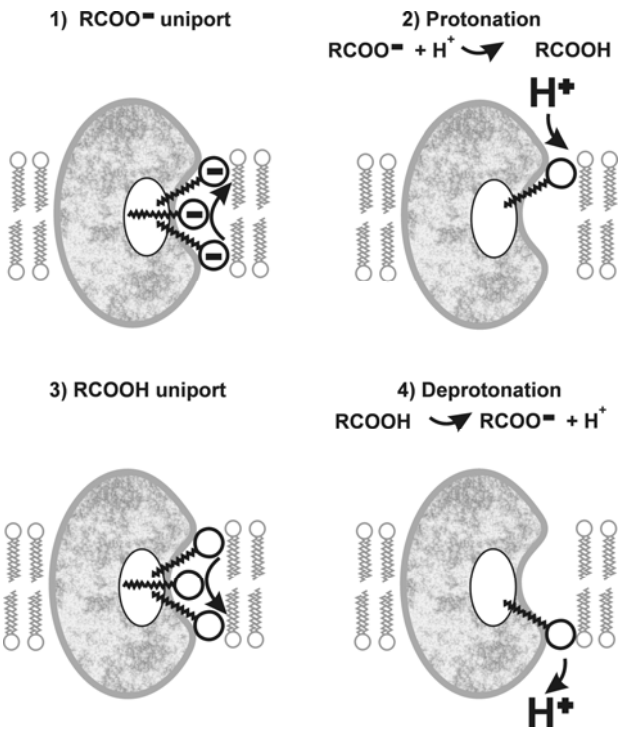

Fig. 1. Fatty acid wobbling mechanism of uncoupling protein mediated uncoupling. Schemas depict the four steps of fatty acid wobbling mechanism suggested by Fedorenko *et al.* (2012).

During the decades of UCP research, mutually incompatible models for uncoupling mechanism have been developed (Skulachev 1991, Garlid *et al*. 1996, Ježek *et al*. 1998, Klingenberg and Echtay 2001, Krauss *et al*. 2005, Cannon *et al*. 2006), which had to include the indisputable facts that the function of all UCPs is initiated by free fatty acids (FFAs). Nevertheless, models that considered UCP as a protonophore had viewed FFA function as a simple facilitation of H^{+} flux into the entry of a "H^{+} channel" (Klingenberg and Winkler 1985, Winkler and Klingenberg 1992, 1994, Gonzalez-Barroso *et al*. 1998, Klingenberg and Huang 1999), though no amino acid residues that would constitute such a channel were ever found. The second model, originally expressed by Skulachev (1991), as fatty acid cycling hypothesis, was in fact developed for all members of SCL25 family and predicted that when an anion carrier *via* its anionic pathway may (even if accidentally) conduct anionic fatty acid (FA), then, spontaneous return of protonated FA *via* the lipid bilayer ensures the H^{+} flux. The FA cycling model has been supported by our numerous studies.

However, recently, using a patch clamp technique to investigate the transport mechanism of UCP1 in native environment of brown adipose tissue mitochondria, a study by Fedorenko *et al.* (2012) has

confirmed that all prerequisites published by Ježek and Garlid (1990) two decades ago were valid (see also Strieleman *et al*. 1985a,b, Ježek *et al*. 1990b, 1994, 1996, 1997a,b, 1998, 2004, Murdza-Inglis *et al*. 1991, Garlid *et al*. 1996, 1998, 2000, 2001, Ježek and Borecký 1998, Jabůrek *et al*. 1999, 2003, Jabůrek and Garlid 2003, Žáčková *et al*. 2003). These patch clamp results together with fatty acid binding studies supported a modified FA cycling model, where FA is not detaching from the protein but is all the time bound to the UCP binding site, from which it alternatively exposes anionic group to the *cis* and *trans* side of the membrane (Fedorenko *et al*. 2012). Thus all premises of the original FA cycling model (Skulachev 1991) were fulfilled, but one, i.e. that FA does not diffuse out of the protein binding site. Such a hindered diffusion has been indicated by our previous EPR studies of UCP1 using 5-DOXYL-stearic acid (Ježek and Freisleben 1994, Ježek *et al*. 1995).

Fedorenko *et al*. (2012) suggested that FA anions are moved within UCPs from *cis* to *trans* side of the membrane so that the tail still interacts with the protein, nevertheless anionic COO^- group is exposed at both sides (Fig. 1). After movement to the *trans* side, protonation occurs and protonated FAs are internalized into the *cis* side by the analogous but counter-directional way and thus carry a proton across the membrane. Such a "local FA cycling" or "wobbling" mechanism cannot proceed with so-called inactive FAs (Ježek *et al*. 1997a,b) or long chain or short chain alkylsulfonates (Garlid *et al*. 1996). The resulting uncoupling would continue until all free FAs are metabolized or removed from IMM by binding to cytosolic FA binding proteins or mitochondrial components that have greater affinities for FAs (Ježek *et al*. 1998).

Moreover, we have shown that UCP2 transports more readily polyunsaturated FAs (PUFAs; Žáčková *et al*. 2003) and hydroperoxy FAs (Jabůrek *et al*. 2004) using recombinant purified UCP2 reconstituted into liposomes or black lipid membranes (Beck *et al*. 2007).

On and off switching of UCP2 function

The extent of UCP's activation is not only governed by FFAs but also owing to the state of inhibition by purine nucleotides (Beck *et al*. 2007). The absolute protein amounts, that may be instantly regulated, serve as a basic parameter for rough estimation of UCP functional relevance in a given tissue under given physiological conditions. It has been reported that lipid peroxidation products, e.g. 4-hydroxy-2-nonenal, may also act as enhancers of UCP-mediated uncoupling by chemical modification of UCPs (Echtay *et al*. 2002a,b, 2003). However, recent study of Fedorenko *et al*. (2012) showed that 4-hydroxy-2-nonenal has no effect on the FFA-dependent H^+ transport mediated by native UCP1.

Moreover, Fedorenko *et al*. (2012) provided further characteristics of FFA-mediated initiation of UCP uncoupling in revealing that it is the nascent FFA, cleaved off phospholipids within the membrane, which preferentially interacts with UCP. This claim, if found accurate, may explain numerous published unsuccessful attempts to elucidate FA role when simple FFA additions were made (e.g. Cunningham *et al*. 1986, Couplan *et al*. 2002, Galletti *et al*. 2009). Already in 2004, we have considered that mitochondria-localized phospholipases A2 (PLA2) can suit such a role (Jabůrek *et al*. 2004). We have originally considered that due to reported preferences of certain PLA2s, probably hydroperoxy-FFAs are cleaved off and initiate UCP2-mediated uncoupling (Skulachev and Goglia 2003). However, Ca^{2+}-independent phospholipase A2 isoform γ (PNPLA8 subfamily of phospholipases A2) has been identified which cleaves not only fatty acid residues from the *sn*-2 positions which are mostly unsaturated, but also from *sn*-1 positions, hence unsaturated FFAs are cleaved (Murakami *et al*. 2011). Recently, we have demonstrated in heart (Ježek J. *et al*. 2010), lung and spleen (Jabůrek *et al*. 2013) and even in pancreatic β-cell mitochondria (Ježek, Dlasková, Jabůrek, *et al*., unpublished) that mitochondrial iPLA2γ (mt-iPLA2γ) is activated by ROS, most probably directly by H_2O_2 (Zelenka, Jabůrek, *et al*., unpublished) and may provide FFAs for either ADP/ATP carrier plus residual UCPs in the heart mitochondria or for abundant UCP2 in lung, spleen and β-cell mitochondria (Fig. 2). The consequent synergy of H_2O_2-activated mt-iPLA2γ and UCP2 thus forms a feedback downregulation of mitochondrial ROS production and may protect against oxidative stress *in vivo*.

We may conclude that besides the acute switch-on of UCP2 expression and maybe concomitant partial suppression of its degradation, redox activation of mt-iPLA2γ is required for UCP2 function. This mechanism may explain even the series of studies when the increase in mitochondrial superoxide production was interpreted as direct UCP upregulation by superoxide or *via* 4-hydroxy-2-nonenal (Echtay *et al*. 2002a,b, 2003).

In addition to the regulation of UCP2 by the redox state *via* mt-iPLA2γ, a direct redox-sensitive modification of UCPs is not ruled out. A reversible

glutationylation was suggested to act as a control switch for UCP2- and UCP3-dependent uncoupling (Mailloux *et al.* 2011). Thus glutathionylation may enhance GSIS and, conversely, increase in mitochondrial ROS was found to deglutathionylate and activate UCP2 and consequently impede GSIS (Mailloux *et al.* 2012). Glutathionylation status of UCP2 thus may contribute to the regulation of GSIS.

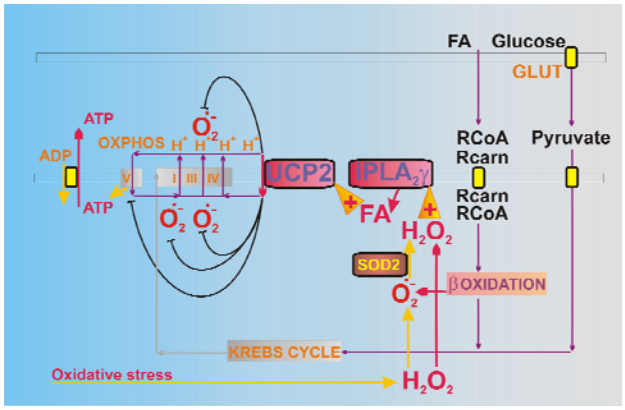

Fig. 2. Synergy of UCP2 and iPLA2γ providing an antioxidant role. Schema depicts the role of UCP2 and mt-iPLA2γ in pancreatic β-cells. For explanations see text.

Evidences that UCP2 attenuates mitochondrial ROS generation

Previously, an antioxidant role for UCP2 has been scarcely demonstrated *in vivo* (Nègre-Salvayre *et al.* 1997, Arsenijevic *et al.* 2000). For example, Duval *et al.* (2002) have shown that UCP2-mediated uncoupling in endothelial cells is even able to decrease extracellular ROS in co-incubated low-density lipoproteins (LDL). 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).

Recently, we have demonstrated for the first time UCP2-mediated suppression of mitochondrial superoxide production *in vitro* (Jabůrek *et al.* 2013). We have shown that mt-iPLA2γ and UCP2 act in concert to protect against oxidative stress in isolated mitochondria and extended this finding to protein carbonylation in lung and spleen tissue (unpublished). The revealed feedback downregulation of oxidative stress is provided by the synergic action of H_2O_2- (or TBHP-)activated mt-iPLA2γ and UCP2, because elimination of either protein prevents synergy. Thus, synergy of H_2O_2-activated mt-iPLA2γ and UCP2 protects against oxidative stress *in vivo* (Fig. 2). In pancreatic β-cells, such FFA feedback downregulation of ROS production (Ježek, Dlasková, Jabůrek, *et al.*, unpublished) is related to the early stages of lipotoxicity which is protected until a certain threshold during the onset of progressive pathology is reached.

The UCP2 role in pancreatic β-cells

Antioxidant role

Let us further focus on UCP2 antioxidant role in pancreatic β-cells. Likewise in other cell types, UCP2 may exert an important antioxidant role in β-cells while preventing excessive superoxide formation within the respiratory chain (Robson-Doucette *et al.* 2011). In pancreatic β-cells it has been observed that UCP2-mediated mild uncoupling decreases the yield of ATP from glucose (Chan *et al.* 2001, Zhang *et al.* 2001). Further studies suggested superoxide activation of UCP2-mediated uncoupling on the basis of observation of elevated $\Delta\Psi_m$ in islets treated with a superoxide dismutase (SOD) mimetic manganese [III] tetrakis (4-benzoic acid) porphyrin (MnTBAP) or overexpressing MnSOD, absent in islets from UCP2 KO mice (Krauss *et al.* 2011). Upon presumed inhibition of UCP2-mediated uncoupling by genipin, $\Delta\Psi_m$ increased in wild type islets but not in UCP2 KO islets (Zhang *et al.* 2006). UCP2 overexpression in INS-1 cells attenuated IL1β-induced ROS formation (Produit-Zengaffinen *et al.* 2007). With UCP2 silencing, a mild uncoupling in mitochondria isolated from INS-1E cells was linked to UCP2, while accounting for up to 30 % of H^+ leak (Affourtit and Brand 2008). UCP2-mediated uncoupling was detectable also in intact INS-1E cells as compared to those silenced for UCP2 (Affourtit *et al.* 2001). In turn, Galletti *et al.* (2009) could not demonstrate any effect of UCP2 overexpression on mitochondrial coupling in INS-1 cells, neither after oleate addition. The chronic absence of UCP2 in UCP2 KO mice of three highly congenic strain backgrounds caused oxidative stress reflected by decreased GSH/GSSG ratio in blood or examined tissues while their islets had elevated levels of antioxidant enzymes and increased nitrotyrosine content (Pi *et al.* 2009). Pancreatic β-cells from UCP2 KO mice had chronically higher ROS when compared to wt mice (Lee *et al.* 2009). Mice with selective knock-out of UCP2 in pancreatic β-cells (UCP2BKO mice) exhibited somewhat increased glucose-induced $\Delta\Psi_m$ (Robson-Doucette *et al.*

2011). UCP2BKO mice had also elevated intracellular ROS levels as determined by DCF (Robson-Doucette *et al*. 2011). These results comply with the antioxidant function of UCP2-mediated mild uncoupling. UCP2 may also modulate redox signaling, if could be effectively switched on and off.

Tuning of redox balance and participation in redox signaling

Cytosolic ROS sources in pancreatic β-cells were reviewed by us recently as well as concomitant redox information signaling (Ježek *et al*. 2012). Hypothetically, redox signaling during apoptosis initiation may reflect the important role of UCPs in immune cells (Carroll *et al*. 2005, Adams *et al*. 2008a,b). As already described above, mild uncoupling may tune mitochondrial ROS production and thus participate in redox regulations. In turn a reversible glutathionylation or glutaredoxin-2 upregulation (Mailloux *et al*. 2013) may act as a control of UCP-participation in redox signaling.

Uncoupling, however, also promotes mitochondrial fission, i.e. disintegration of mitochondrial network into small objects containing usually one or several nucleoids of mtDNA (Tauber *et al*. 2013) that can be theoretically more readily degraded within them, under certain circumstances by mitophagy, a mitochondria-specific authophagy (Gomes and Scorrano 2013). Mitophagy, however, when exerted in optimum frequency might be beneficial to cell, namely due to degradation of oxidatively-modified cell constituents, hence mild uncoupling promoting "mild" mitophagy should be physiological.

Autophagy is a „self-eating" process allowing control on degradation of either insoluble protein aggregates or damaged organelles (mitochondria, endoplasmatic reticulum, etc.) in lysozomes (Murrow *et al*. 2013) and is involved in lipid control (Christian *et al*. 2013). Dysregulated autophagy and mitophagy is a generally accepted mechanism that is involved in diseases related to aging, including Type 2 diabetes mellitus (T2DM, Horan *et al*. 2012, Hubbard *et al*. 2012). Synergically with glucose FFAs block autophagic turnover in pancreatic β-cells (Las *et al*. 2011). Impairment of insulin secretion was also associated with deficient autophagy in animal model, when marked decrease in autophagy-cascade-related proteins like microtubule-associated protein 1 light chain 3 (LC3/Atg8), LC3 II/I ratio and autophagy-related protein 7 (Atg7) or lysosomal-associated membrane protein 2 (LAMP2) together with increase of sequestosome-1 (SQSTM1/p62) and polyubiquitinated protein aggregates were recorded in aged rats (Liu *et al*. 2013). Mice with β-cell-specific Atg7 deletion showed reduced β-cell mass and pancreatic insulin content, however, they have not developed diabetes. Upon breeding these mice with obese (*ob*/*ob*) mice, animals became diabetic (Quan *et al*. 2012). Reduced autophagy may lead to a significant susceptibility to additional long-acting injury of β-cells in elderly-like hyperlipidemia and even moderate hyperglycemia due to the insulin resistance, both related to diet-induced obesity. In turn, UCP2-mediated mild uncoupling can be beneficial to promote a slight fission of mitochondrial network and contribute to the physiologically required intensity of mitophagy. In pancreatic adenocarcinoma cells, UCP2 inhibition or silencing lead to ROS increase and expression of autophagic marker LC3 II (Dando *et al*. 2013).

Regulation of glucose-stimulated insulin secretion (GSIS)

The intimately specific feature of pancreatic β-cells lies in glucose sensing through the OXPHOS (Ashcroft and Rorsman 2012). Respiration and OXPHOS rates, leading to a certain ATP/ADP ratio, are governed by the availability of glucose, whereas in most other cell types, cell demand dictates respiration/metabolism rates and the ATP/ADP ratio. It is because of a specific enzyme/regulation pattern of β-cells (Ježek *et al*. 2012). At first, unlike in numerous other cell types, pyruvate cannot be diverted towards lactate dehydrogenase for lactate formation in β-cells. Consequently, glucose cannot be metabolized by aerobic glycolysis, which provides so-called Warburg phenotype in cancer cells and under physiological cell responses to hypoxia and other adaptations (Ježek *et al*. 2010, Smolková *et al*. 2011). Thus nearly 100 % of glucose is metabolized by OXPHOS in β-cells (likewise in hepatocytes and numerous differentiated OXPHOS cells). The pattern of pyruvate dehydrogenase kinase (PDK) genes is surely responsible for this (Ježek *et al*. 2012). Thus, β-cell PDK1 and PDK3 are "constitutively blocked", and PDK2 is "inefficient" so that it does not phosphorylate PDH E1α subunit of pyruvate dehydrogenase (PDH), hence does not inhibit its activity. At low basal glucose, PDH is 90 % active, whereas at maximum glucose PDH is inhibited only by 22 %. Also hexokinase IV (glucokinase) in β-cells is not inhibited by glucose-6-phosphate, as in e.g. skeletal muscle cells. The lack of such a feedback inhibition of glycolysis directly connects

glycolysis to pyruvate. Finally, the human glucose transporter GLUT1 or rodent GLUT2 are not dependent on insulin, so glucose in β-cell cytosol is proportional to bloodstream glucose. This is a perfect setting for a sensor.

Consequently, glucose metabolism in β-cells is finely adjusted to the blood glucose levels (Merglen *et al*. 2004). At starvation with ~3 mM glucose levels, β-cell respiration is relatively low, as well as the intensity of ATP synthesis, corresponding to the established state-$3_{[Glc=3mM]}$ (Liang *et al*. 1997, Porterfield *et al*. 2000, Špaček *et al*. 2008). The $\Delta\Psi_m$ is still lower than would be at state-4 with 3 mM glucose. Increasing glucose intake into β-cells, may increase up to OXPHOS-saturating ~12 to 15 mM glucose, when maximum OXPHOS takes places with the established state-3_{max}, maximum respiration and maximum $\Delta\Psi_m$ (Špaček *et al*. 2008). The resulting increased ATP/ADP ratio in the cell cytosol initiates closure of plasma membrane ATP-sensitive K^+ channels (Ashcroft and Rorsman 2012), leading to plasma membrane depolarization and opening of voltage-sensitive Ca^{2+} channels. Increased cytosolic Ca^{2+} initiates insulin granule exocytosis. It has been hypothesized that β-cells maintain a relatively high [ATP]/[ADP] value even in low glucose and that glucose metabolism leads to dramatically decreased free ADP with only modestly increased ATP (Fridlyand and Philipson 2004). If a high [ATP]/[ADP] ratio exists even at low glucose levels, as a result, the total adenine nucleotide concentration is unchanged during a glucose-induced elevation. GSIS was also reported to be modulated or accelerated by other metabolic pathways related to mitochondria, such as phosphocreatine shuttle, additional Ca^{2+} signaling due to glutamate metabolism (Maechler *et al*. 2006, Casimir *et al*. 2009), citrate export (Joseph *et al*. 2006), phosphoenolpyruvate (Stark *et al*. 2006) and pyruvate cycling (Heart *et al*. 2009, Jitrapakdee *et al*. 2010). A common denominator in these modulations is NADPH, the role of which on insulin secretion has yet to be established. Overall, GSIS possesses also a component due to the autocrine function of insulin. The UCP2 function in GSIS regulation has been firmly established (Chan *et al*. 2001, Zhang *et al*. 2001). Now we hypothesize, that due to UCP2 participation in redox regulations and due to redox homeostasis effects on the NADPH content, UCP2 may exert the second arm for GSIS fine tuning, the redox regulation arm.

Future perspectives

Due to its complex health and economic sequels as well as steadily increasing prevalence, Type 2 diabetes mellitus represents one of the serious burdens of the 21th century. Its pathogenesis is complex and different factors may prevail in individual cases. The typical feature of progressed T2DM is insulin resistance as well as β-cell dysfunction (Ashcroft and Rorsman 2012, Ježek *et al*. 2012). In future research it will probably be established whether T2DM is an inevitable disease and whether one may develop a strategy to highly retard or completely exclude the pathological outcomes of progressive self-accelerating oxidative stress and nitrosative stress and concomitant dysregulated information signaling. The emerging role of redox signaling in GSIS and processes of molecular physiology of pancreatic β-cells need to be elucidated as well. Unfortunately, neither targeted antioxidants might be able to defeat T2DM, since they simultaneously disrupt the inherent physiological redox signaling. Perhaps more focused strategies on yet unknown mechanisms will help to defeat T2DM world epidemics. Molecular research on UCP2 roles during diabetes development may significantly contribute to this goal.

Conflict of Interest

There is no conflict of interest.

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