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Review

Uncoupling mechanism and redox regulation of mitochondrial uncoupling protein 1 (UCP1)

Petr Ježek[a,1], Martin Jabůrek[a], Richard K. Porter[b,*]

[a] Department of Mitochondrial Physiology, No. 75, Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic
[b] Trinity Biomedical Sciences Institute, Trinity College, Dublin, Ireland

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ABSTRACT

Brown adipose tissue (BAT) and brown in white (brite) adipose tissue, termed also beige adipose tissue, are major sites of mammalian nonshivering thermogenesis. Mitochondrial uncoupling protein 1 (UCP1), specific for these tissues, is the key factor for heat production. Recent molecular aspects of UCP1 structure provide support for the fatty acid cycling model of coupling, i.e. when UCP1 expels fatty acid anions in a uniport mode from the matrix, while uncoupling. Protonophoretic function is ensured by return of the protonated fatty acid to the matrix independent of UCP1. This mechanism is advantageous for mitochondrial uncoupling and compatible with heat production in a pro-thermogenic environment, such as BAT. It must still be verified whether post-translational modification of UCP1, such as sulfenylation of Cys253, linked to redox activity, promotes UCP1 activity. BAT biogenesis and UCP1 expression, has also been linked to the pro-oxidant state of mitochondria, further endorsing a redox signalling link promoting an establishment of pro-thermogenic state. We discuss circumstances under which promotion of superoxide formation exceeds its attenuation by uncoupling in mitochondria and throughout point out areas of future research into UCP1 function.

1. Introduction

During the process of oxidative phosphorylation, a protonmotive force (Δp), consisting of two theoretical components, electrical potential difference ($\Delta\Psi_m$) and proton chemical potential difference (expressed as ΔpH), is established across the inner mitochondrial membrane (IMM). Peter Mitchell's chemiosmotic theory clearly demonstrated that Δp is formed by the pumping of protons by the respiratory chain complexes, whereas Δp is consumed by the proton backflow via the F_O moiety of the ATP synthase and by the $\Delta\Psi_m$- and ΔpH-dependent substrate or ion translocation and enzyme-dependent processes (e.g. nucleotide transhydrogenase). Balance between Δp formation and consumption by the ATP synthase is termed coupling. In contrast, uncoupling describes the situation when additional H^+ backflow exists, consuming Δp without providing ATP, transport, or enzymic reaction [1]. The futile cycling of protons being pumped across the IMM followed by the short-circuit, that is the proton backflow across the IMM, results in heat production.

A diffusion based, Δp-dependent, basal proton backflow determined by the inherent permeability of IMM is termed proton leak [2,3]. Specialized mitochondrial uncoupling proteins (UCPs), with tissue specific expression, provide an additional and regulable “proton leak” [4,5]. This latter UCP catalyzed proton leak is regulated by levels of expression of the UCP, purine nucleotides, free fatty acids and possibly by posttranslational modification of the UCPs [6–10]. The focal point of this review is uncoupling protein 1 (UCP1), the archetypal uncoupling protein. UCP 1 is primarily associated with brown adipose tissue (BAT) and is the best characterized of the UCPs. Importantly, of the UCPs, UCP1 seems unique in its thermogenic role. When active, UCP1 leads to non-shivering thermogenesis in BAT [11–15] and in beige adipocytes (also termed “brown in white”, i.e. brite adipocytes), the latter being located within non-thermogenic white adipose tissue (WAT) [16–20].

Over the decades, however, several lines of enquiry have been developed to explain the uncoupling ability of UCP1. A simple view would

Abbreviations: GSH GSSG, reduced, oxidized glutathione; HIF, hypoxia–induced factor; iPLA2γ, calcium-independent phospholipase A2γ; NOS, nitric oxide synthase; NOX, NADPH oxidase, EC 1.6.3.1; OXPHOS, oxidative phosphorylation; RC, respiratory chain; SOD, superoxide dismutase, EC 1.15.1.1; ROS, reactive oxygen species

* Corresponding author at: School of Biochemistry & Immunology, Room 6.08, Trinity Biomedical Sciences Institute, Trinity College Dublin, Pearse Street, Dublin 2 (D02R590), Ireland.
E-mail addresses: jezek@biomed.cas.cz (P. Ježek), rkporter@tcd.ie (R.K. Porter).
[1] Dept. No.75, Mitochondrial Physiology, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14,220 Prague 4, Czech Republic.

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be that the uncoupling, and thus thermogenic capacity of UCP1 is due simply to its abundance in a tissue [4]. However, when UCP1 was overexpressed in tissues other than BAT, such as in the heart [21], it did not uncouple mitochondria nor did it facilitate heat production. The major reason for this lack of uncoupling seems to be inhibition of UCP1 activity by purine nucleotides. UCP1, like the other UCPs, contains an outer facing cavity, and occupation of that site with purine nucleotide di- and triphosphates, in the absence of Mg^{2+}, results in UCP1 inhibition [22–27]. Thus, early studies of BAT and brown adipocytes recognized the specific "thermogenic" environment of this tissue and its mitochondria [28–31]. This specific "thermogenic" environment consists not only of having *i*) UCP1 expressed in rather high amounts [5], but also *ii*) diminished amounts of ATP-synthase [32,33] *iii*) appropriate metabolic pathways to yield reducing equivalents [34], *iv*) the existence of a specific information signalling pathways [35] and *v*) adequate biogenesis of BAT [36]. In this review, we aim to summarize the properties of UCP1 common to other UCPs plus the intracellular conditions required for thermogenic mitochondrial function. We will also briefly comment on recent advances in the mechanism of activation of UCP1 and the role played by redox signalling in that mechanism and in biogenesis of BAT.

2. Uncoupling mechanism of mitochondrial uncoupling protein UCP1

2.1. Fatty acid cycling mechanism

When it comes to the fundamentals of bioenergetics, a pertinent question arises in relation to UCPs, which is, how much of the protonmotive force (Δp) has to be dissipated in order to release sufficient heat to raise the temperature of BAT or other tissues? One may also ask whether the other UCP isoforms possess the ability to dissipate Δp below such a threshold? In looking for the answer to these questions, we should recall the experiments reporting the kinetic range over which UCP1 uncoupling in BAT mitochondria occurs, that being between 220 mV and 166 mV, a range of ~55 mV [37,38]. UCP2 has also been demonstrated to catalyze a regulable "proton leak" and significant UCP2 protein is expressed in spleen, lung, stomach, white adipose tissue and immune cells [5]. In contrast to UCP1, estimates for the maximal uncoupling range for UCP2 in lung mitochondria were 12.5 mV [37,38]. The maximum activity of UCP1 has been evaluated as 20 μmolH^+/min *per* mg UCP [39–41]; i.e. 333 nmol H^+/s *per* mg UCP, which is equal to a turnover number of 11 s^{-1}. Reconstitution of UCP2 into liposomes and black lipid membrane (BLM) show a similar V_{max} for UCP2 as for UCP1 [42,43]. Given the fact that UCP2 abundance is two orders of magnitude lower than that of UCP1, the range over which UCP2 uncouples reflects the relative abundance of UCP2. In conclusion, the amount of uncoupling protein within a thermogenic/non-thermogenic tissue is crucial to thermogenic capacity. However, UCP activity is also a factor.

Thermogenesis in BAT has been linked to metabolism of fatty acids (FAs) and early studies also recognized that FAs are obligatory for the activity of UCPs studied in proteoliposomes [40,41,44–46] and in BLMs [43,47]. This absolute requirement of FAs for UCP function has also recently been confirmed for UCP1 in patch-clamping experiments on IMM of BAT mitochondria [48].

In order to explain the mechanism of UCP1 action, the FA cycling hypothesis was originally proposed by Skulachev [49] and modeled in detail by Garlid and co-workers [39,46,50,51]. Since then the FA cycling model has been supported by numerous experiments and the structural basis for the model, i.e. namely the proposed evidence for the existence of the peripheral FA binding sites, has been supported by several laboratories [27,52,53]. Furthermore, the FA cycling hypothesis concept is clearly supported by the existence of synthetic small molecules - uncoupling protein mimics, which are able to perform FA-activated H^+ translocation in phospholipid bilayers [54]. There is also a plausible hypothesis that FA cycling proceeds predominantly within the specific portion of the IMM forming the rich enfolded cristae, the intracristal space (ICS) membrane [55].

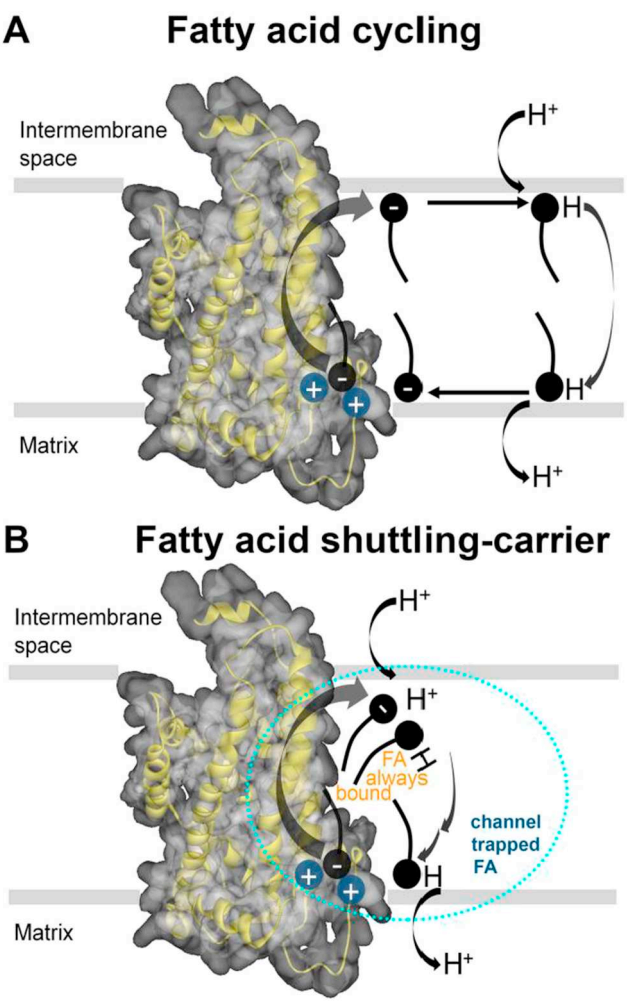

Fig. 1. UCP1 as a fatty acid anion uniporter.
A) Fatty acid cycling mechanism – The UCP-catalyzed protonophoretic cycle represents the most plausible mechanism of function for all UCPs [55]. Fatty acid (FA) anions diffuse laterally within the membrane to reach a subsurface peripheral binding site on UCP1 near the matrix [61] where it binds specifically to basic residues (depicted as blue dots) [27,52,53]. The $\Delta\Psi_m$ across the inner mitochondrial membrane drives the carboxylate head group through an electrostatic path composed of basic residues both inside and outside the UCP cavity [70], resulting in a transport of FA to the other side of the membrane. The FA anion diffuses laterally away from UCP, where it is protonated. Protonated FA diffuses rapidly back across the membrane to deliver protons electroneutrally to the matrix by a spontaneous flip-flop mechanism [67], completing the cycle.
B) Fatty acid shuttling-carrier mechanism considers UCP1 protein as a "carrier" where FA shuttles back and forth across the inner mitochondrial bound to UCP1 (wobbling) [48]. This mechanism differs from the fatty acid cycling mechanism in that the FA molecule cannot diffuse away from the UCP protein and stays bound to the protein, in an unspecified way, while being exposed to the *cis* or *trans* side of the membrane either as anion or after protonation. In this case, both anionic and neutral protonated FA are carried through the UCP1 protein. However, the mechanism seems unlikely since the actual fatty acid binding site has been verified to face the lipid bilayer [27,52,53].

The FA cycling mechanism assumes that UCP1 and other UCPs are not protonophores but fatty acid anion uniporters (Fig. 1) [39,46,50,51]. The anionic FA^- is translocated by UCP from the matrix surface of the ICS membrane toward its ICS surface, thus consuming the established Δp. After the UCP-mediated translocation, the anionic FA^-

is protonated. At physiological pH, the protonated, neutral FA, can easily flip back across the lipid bilayer of ICS membrane to its matrix surface. As a result, uncoupling and concomitant Δp consumption is not taking place within the UCP protein, but is facilitated by spontaneous diffusion of protonated FAs across the phospholipid membrane (flip-flop mechanism). The continuation of the cycle is ensured by FA^- anion transport, which is mediated by UCP. The overall FA cycling thus leads to apparent UCP-mediated H^+ translocation [46,56–58].

Crucial support for the FA cycling hypothesis comes from the observations of the inability of so-called "inactive FAs" (such as long chain dicarboxylic acids, FAs with bulky or polar groups at the ω end) to induce the protonophoric ability of UCP1 [56,59]. In contrast to the fatty acid cycling model, the protonophoric model postulates that the role of fatty acids is to insert into UCPs providing their α-carboxyls for proton conductance through UCPs [34,44]. If the protonophoric model is correct then bulky or polar groups at the ω end of long chain fatty acids should not affect function. Since these FAs are also unable to flip-flop across the lipid bilayer in a protonated form, the observation of the absence of UCP1 activation with "inactive FAs" strongly supports FA cycling hypothesis. An apparent exception to the "inactive FAs" came in the form of perfluro-octanesulfonate which was reported to activate UCP1 protonophoric function [60], contrary to a wide array of alkyl-sulfonates which do not activate [46,52,61]. However, a disturbance of the lipid bilayer by perfluro-octanesulfonate is thought to account for the nature of this exception [62].

Interestingly, Echtay et al. [63] looked at the fatty-acid-dependent H^+ transport activity in *Escherichia coli* expressed UCP1 reconstituted into liposomes and determined that coenzyme Q (CoQ) was an obligatory cofactor for UCP1 function. They also found that the H^+ transport was highly sensitive to purine nucleotides in this reconstituted system, and activated only by oxidized CoQ (ubiquinone) but not reduced $CoQH_2$ (ubiquinol). Moreover, they established that H^+ transport of native UCP1 correlated with the endogenous CoQ content [63]. This result was challenged by Jabůrek and Garlid [41], who found that reconstituted recombinant UCP1 is unaffected by oxidized CoQ and concluded that CoQ is not a cofactor of UCP-mediated proton transport [41]. This controversy raised the question of the many challenges associated with the isolation and refolding of bacterially expressed membrane proteins. Specifically, it led to speculation that the need for CoQ might have reflected specific experimental conditions used in Echtay et al. [63] and that the addition of CoQ (and/or the associated solvent) might have promoted structural changes that enabled misfolded UCP1 to support FA-dependent H^+ transport [41].

In support of the lack of the effect of CoQ on UCP1 activity, it was found that oxidized CoQ was not required for proton conductance by UCP1 heterologously expressed in yeast mitochondria [64]. In this study, *Saccharomyces cerevisiae* mutant strains lacking CoQ and expressing UCP1 were used to determine whether CoQ was required for UCP1 function. Because the activity of UCP1 was similar in both CoQ-containing and CoQ-deficient yeast mitochondria, the authors concluded that CoQ is neither an obligatory cofactor nor an activator of proton transport by UCP1 when it is expressed in yeast mitochondria [64]. Further detailed studies on the effect of CoQ using heterologously expressed rat UCP1 in yeast mitochondria, as well as native UCP1 in rat BAT, indicated that the endogenous ubiquinone redox state had no effect on fatty acid-induced UCP1 activity in the absence of GTP, and can only regulate this activity through sensitivity to inhibition by the purine nucleotide [65]. Namely, the CoQ redox state-dependent alleviation of UCP1 inhibition by the purine nucleotide was observed at a high CoQ reduction level (exceeding 85% of total CoQ). Even though these findings indicate that UCP1 sensitivity to GTP can be regulated by the redox state of membranous CoQ, they provide no mechanism, explaining how ubiquinol could directly interact with UCP1. Instead, the correlation between the mitochondrial redox state, CoQ redox state and the ability of purine nucleotides to inhibit FA-dependent H^+ transport by UCP1 may be attributed to the direct oxidative post-translational modification of UCP1, such UCP1 Cys253 sulfenylation, as demonstrated by Chouchani et al. [66].

An alternative model to the fatty acid cycling model was recently hypothesised for UCP1 function. It was proposed that both FA^- anions and neutral protonated FAs are transported by UCP1 and that neither FA form diffuses out of the protein during this mechanism [48]. This FA shuttling mechanism is however unlikely from a thermodynamics point of view [55] and is incompatible with the revealed structures of UCPs, specifically with the existence of the surface FA binding site. In this model, FAs does not diffuse out of UCP to the phospholipid bilayer, but remains bound to the UCP by hydrophobic interactions. Protonated FAs thus carry (shuttles) H^+ back to the matrix while still attached to the protein. However, the hydrophobic interaction does not allow FAs to reach thermodynamic equilibrium between UCP and the phospholipid bilayer. Given the extremely fast rate of protonated FAs movement across the phospholipid bilayers [67] and the relatively slow transport of ionized FAs by UCPs [40,46], the proposed hydrophobic interaction of FAs with the UCP would have to be orders of magnitude stronger than the hydrophobic interaction of FAs with the membrane, a key fact that undermines this model. Furthermore, the known dependence of UCP activity on specific fatty acid chain length [42,68] is also inconsistent with the FA shuttling mechanism [48] and, more consistent with the FA cycling hypothesis.

Other models, pronouncing the net protonophoric function of UCP1, are still debated [69], despite the experimental evidence to the contrary, from studies on UCP2 structure and FA interaction with UCP2 [55]. Furthermore, the interaction of UCP1 with FAs have been studied using NMR [53]. The authors clearly demonstrated that FA directly binds UCP1 at the interface between transmembrane α-helix H1 and H6. The paramagnetic relaxation enhancement together with molecular dynamics simulation, demonstrated that the FA carboxylate group is attracted by basic residues on UCP1 near the matrix side, whereas the hydrophobic FA tail fits into the H1/H6 groove. The FA binding is thus similar to the FA binding to UCP2 described previously [52,70]. Also, mutagenesis experiments carried out on UCP2 and UCP1 yield similar outcomes [52] – decreased UCP-mediated H^+ flux at substitutions on FA interacting residues. In conclusion, structure/functional relationships support the identical uncoupling mechanism by UCP1 and UCP2 and support the FA cycling mechanism.

2.2. Nascent fatty acid requirement for uncoupling protein function and synergy with mitochondrial phospholipases

Recently, another detail was revealed for UCP1 functional activation in vivo, based on the observation that only those fatty acids instantly cleaved by mitochondrial phospholipases induce UCP1-mediated uncoupling [48]. This phenomenon was indicated during studies by direct patch-clamp of UCP1 within its natural IMM environment of BAT mitochondria [48]. A similar phenomenon has been described for UCP2 in INS-1E cells with involvement of redox-activated iPLA2γ in synergy with UCP2, acting as an antioxidant mechanism and redox suppressing regulator [71]. Studies using mitochondria isolated from tissues rich in UCP2 [72] also identified H_2O_2-activated mitochondrial iPLA2γ as the main regulator of the UCP2 activity. Typically, iPLA2γ is activated by H_2O_2 formed from superoxide during FA β-oxidation. Thus, iPLA2γ, by cleaving phospholipids and releasing free FAs, provides the anionic substrates for UCP2-mediated uncoupling. The consequent partial dissipation of Δp initiates a direct feedback attenuation of mitochondrial superoxide production [71–73]. It remains to be established whether during typical FA β-oxidation, which is a hallmark of BAT thermogenesis, iPLA2γ also cleaves free FAs for UCP1, and whether this mechanism supersedes the "classic" mechanism where FAs from lipolysis (not yet esterified to FA-CoA and FA-carnitine for the carnitine cycle) are cycling substrates of UCP1. Speculatively, iPLA2γ might be activated by the same redox signal that leads to sulfenylation of Cys253 on UCP1 [66], as we will discuss later. Our preliminary data

from experiments comparing iPLA2γ KO mice to wild-type mice support the partial contribution of the redox-activated iPLA2γ to the initiation of BAT thermogenesis (Jabůrek et al., unpublished).

2.3. Implications for UCP1 function and regulation based on UCP1 structure

Early studies by Martin Klingenberg had concluded that UCP1 (~32 kDa as a monomer) exists within the IMM as a dimer which is able to bind just a single molecule of nucleotide [74]. Recently, it has been shown that the monomeric UCP1 can exists which binds a single nucleotide [75]. These detergent-free purified UCP1 preparations retained the tightly bound three molecules of cardiolipin *per* monomeric UCP1. The structure of UCP1 is implicitly modeled as based on the crystal structure of the ADP/ATP carrier [76], since the common structural features exist for the whole mitochondrial anion carrier protein, i.e. SLC25, gene family [77]. Alternatively, UCP1 could be modeled analogously to the NMR structure of UCP2 [52,70]. In both cases, critical conserved positively charged residues exist on the protein surface that were demonstrated to bind fatty acids as well as containing an ICS facing binding site (cytosolic orientation) for purine nucleotide binding. In addition, valuable information was attained by Zhu et al. [27] who mapped the nucleotide binding site of UCP1 using atomic force microscopy. By comparing recognition patterns obtained with an anti-UCP1 antibody and the ligand ATP they demonstrated that the ATP binding site can be accessed from both sides of the membrane. However, only binding from cytosolic side leads to the protein conformational changes and inhibition of UCP1. They also calculated that the distance of the binding site from the surface is approximately 1.27 nm, thus providing further valuable structural information about UCP1.

2.4. Posttranslational modifications of UCP1

Detailed identification of post-translational modification of proteins is now available with the advent of advanced mass spectroscopy. Enormous physiological significance has been associated with sulfenylation, acetylation, succinylation and other residue modifications. For example, acetylation of numerous mitochondrial proteins inhibits their function, whereas their deacetylaton, by certain sirtuins, promotes metabolism and oxidative phosphorylation. Among mitochondrial sirtuins, sirtuin 3 is a NAD^+-dependent deacetylase essential for oxidative phosphorylation [78], whereas sirtuin 5 does a similar job while acting as a potent desuccinylase [79]. Among mitochondrial proteins targeted are subunits of the ATP synthase, optic atrophy associated protein 1 (OPA1), and magnesium dependent superoxide dismutase (MnSOD) which are inhibited by acetylation and physiologically activated by sirtuin 3. Similarly enzymes of fatty acid β-oxidation and ketogenesis are activated by sirtuin 5 mediated de-succinylation [80] and, interestingly, sirtuin 4 removes three acyl moieties from lysine residues on enzymes associated with leucine metabolism [81]. Nevertheless, it has to be determined, whether UCPs also possess modified lysine residues.

Sulfenylation of cysteines is another type of posttranslational modification, reported to be employed in transmittance of the redox signalling by thioredoxins, peroxiredoxins or glutaredoxins [82]. Recently, this type of modification has been proposed to be essential for initiation of thermogenesis in BAT. Specifically, Cys253 of UCP1 has been identified as the key regulator of non-shivering thermogenesis, being oxidized to a sulfenyl by reactive oxygen species (ROS) upon norepinephrine stimulation of BAT or brown adipocytes [66]. Criticism of the finding focuses on the mechanistic link between norepinephrine stimulation and the redox changes required to provide sulfenylation [55]. In addition, there would have to be quite a strong redox signal to modify the significant amount of UCP1 present in BAT, therefore one might also expect analogous cysteines of the other carriers of the SLC25 family to be modified, which they are not [69]. Consequently, further research on the signalling pathways involved and the relevance in vivo requires further investigation.

An intriguing speculation on our part, alluded to earlier, that could unify known aspects of UCP1 activation, is the redox dependent activation mechanism which has been proposed for UCP2 [71]. In essence, nascent FAs are cleaved from cardiolipin and phospholipids in the IMM by the redox activated phospholipase iPLA2γ (PNPLA8). The phospholipase provides the substrate for UCP flipping activity, while the long chain fatty acyl carnitines, brought to the matrix for β-oxidation as a result of norepinephrine/PKA- stimulated BAT lipases in the cytosol, provide the substrate for a sufficient ROS burst by ETF:QOR to which other respiratory chain or α-glycerol- phosphate dehydrogenase may also contribute. However, it is important to note that the functional activation of iPLA2γ during norepinephrine stimulation of brown adipocytes has yet to be demonstrated.

2.5. Fatty acid cycling and thermogenesis

How compatible is the fatty acid cycling model with mitochondrial heat production? What is the advantage of the fatty acid cycling model over a simple protonophoric channel (uniport) mechanism when it comes to explaining heat production? One can search for a clue in the mitochondrial ultrastructure of enfolded cristae [83], which are very prevalent in BAT mitochondria. Recent evidence suggests that proton pumps are concentrated within the supercomplexes and are localized in the flatter cristae walls [84]. These supercomplexes pump protons, not to the cytosol per se but, to mostly prolongated intracristal spaces (ICS), oriented perpendicularly toward the cylindrical structure that is made up of the outer mitochondrial membrane (OMM), the (outer) intermembrane space region and the inner boundary membrane (IBM) (Fig. 2). The IBM thus represents an inner side of a cylinder of unfolded IMM, not part of the crista outlets emanating from the IBM and from where junction proteins form joints with the OMM. These cylindrical morphologies form the tubules of the mitochondrial network, which are connected in many tissues, including skeletal muscle and heart. The mitochondrial network undergoes fission and fusion and remains connected when these two processes are balanced or fusion prevails. The membrane network in brown adipocytes has been frequently studied [85,86].

Coupling between the proton pumps and ATP synthesis is, therefore, localized to the ICS and involves two-dimensional (2D) diffusion of H^+ from the flat cristae walls to the cristae edges, where dimers of the ATP-synthase are localized (Fig. 2). Any uncoupling must interrupt this 2D diffusion, otherwise local coupling is intact. The advantage of the FA cycling model over protonophoric channels lies in the fact that in order to be efficient, any presumably proteinaceous channel would have to be very abundant, so as to be able to intersect all paths of 2D diffusion. Such hypothetical H^+ channels should be preferentially located also in the flat cristae walls to scavenge H^+ closest to their outlet sites of supercomplex proton pumps (Fig. 2B). In contrast, the anionic FA^- is able to diffuse in the 2D plane of the ICS-adjacent lipid leaflets of the ICS membrane (a folded portion of IMM), the fatty acids can easily reach the cardiolipin annuli around the protein complexes and after FA^- protonation, i.e. after scavenging the pumped H^+, the neutral FA can flip-flop back to the matrix-adjacent lipid leaflets of ICS membrane. 2D diffusion in the plane of the membrane ensures fast access to the UCP (Fig. 2A). Anywhere on its route within the matrix ICS membrane, surface neutral FAs can be deprotonated which is facilitated by the more alkaline matrix pH. UCP is not localized in the proximity of the ATP synthase, hence fast turnover of uncoupling is ensured by the involvement of numerous FA and probably also by constant FA cleavage from mitochondrial membranes by phospholipase iPLA2γ. Recently Pohl's group have shown that UCP4 is spatially separated from the ATP synthase in neuronal mitochondria [87]. UCP4 is also regarded as a proton transporter activated by fatty acids [88]. The localization of UCP4 at the inner boundary membrane (IBM) is consistent with the view that it dissipates the excessive proton gradient, without affecting

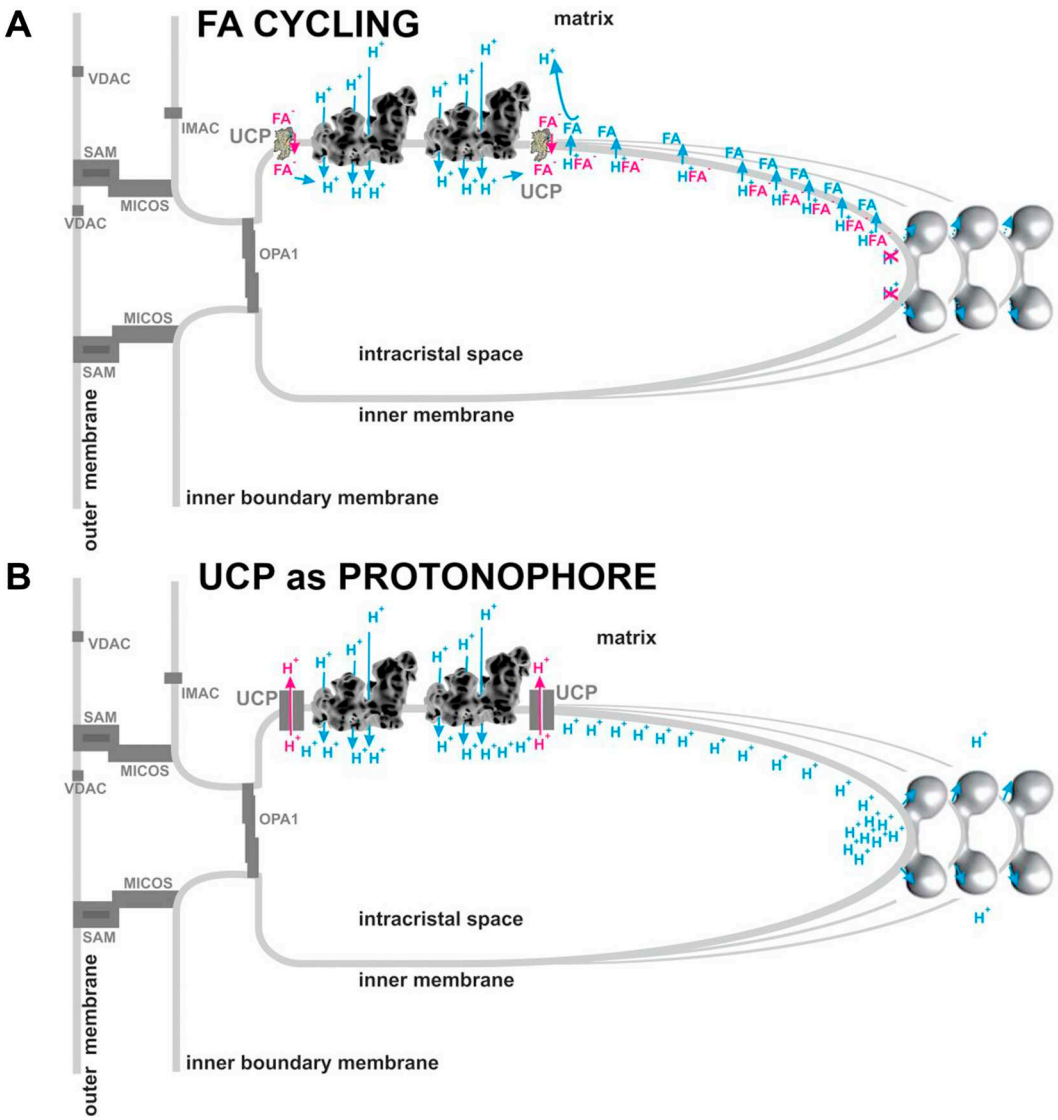

Fig. 2. Advantage of FA cycling for uncoupling of inner mitochondrial membrane cristae at existing 2D proton diffusion.
Coupling between the proton pumps of the respiratory chain supercomplexes (depicted as dark grey clusters in the inner membrane), located at flat cristae regions, and ATP synthase dimers (depicted as light grey bulbus protrusions from the right edge of the inner membrane edge), organized in arrays at the cristae edges, must involve 2D diffusion of H^+ from the flat cristae walls to the cristae edges. The advantage of the (A) FA cycling mechanism over (B) the protonophoric channel mechanism of uncoupling lies in the following:
A) Anionic FAs diffuse in the 2D plane of the membrane to reach the cardiolipin *annuli* around the supercomplexes, where they are protonated by incoming H^+ from H^+pumping. The resulting neutral FAs flips back to the matrix-adjacent lipid leaflets and their 2D diffusion within the membrane ensures their fast translocation to UCP. Essentially, anywhere on their route neutral FAs can be deprotonated, facilitated by the more alkaline matrix pH. Since UCP is not localized in proximity to the ATP synthase dimer, a fast turnover rate of uncoupling is ensured by numerous FA molecules being involved.
B) In contrast, in order to achieve a fast turnover rate of uncoupling, any proton translocating proteinaceous channel would have to be very abundant, so as to be able to intersect along the path of 2D diffusion. Such H^+ channels should also be preferentially located in the inner membrane flat cristae walls to scavenge H^+ closest to their outlet sites of supercomplex proton pumps.
Abbreviations: IMAC – inner membrane anion channel; MICOS – mitochondrial contact site and cristae organizing system; OPA1 – Optic atropy type 1 protein; SAM – sorting and assembly machinery; UCP – uncoupling protein; VDAC – voltage-dependent anion channel.

ATP production. The competition between UCP1 and the ATP synthase was not convincingly shown in BAT, so the possibility exists that UCP1 is also localized at IBM.

3. Brown adipocytes as an ideal environment for promoting thermogenesis

3.1. Norepinephrine stimulation of BAT

Brown adipose tissue (BAT) is formed predominantly by brown adipocytes containing multiple small lipid droplets and high content of mitochondria and it is highly vascularized and innervated so that nearly each cell has its own nerve terminal [11]. BAT tissue also contains preadipocytes with the ability to differentiate into brown adipocytes [89,90]. In rodents small BAT depots are distributed in interscapular, subscapular, axillary, perirenal and periaortic regions. The sympathetic nervous system centrally regulates nonshivering thermogenesis stimulated by norepinephrine via β3- adrenergic receptors in rodents but predominantly by β1- adrenergic receptors in humans [12]. It has long been established that the cold-induced sympathetic stimulation of brown adipocytes activates lipolysis, glucose uptake, as well as mitochondrial biogenesis. This acute norepinephrine stimulation of BAT

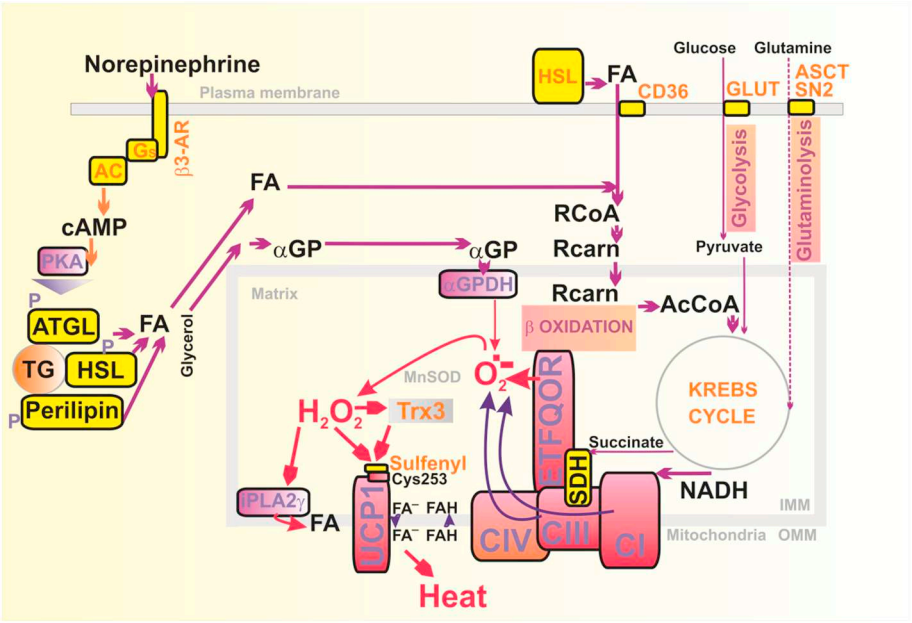

Fig. 3. Nonepinephrine initiation of thermogenesis in brown adipocyte.
The scheme describes the known cascade of events beginning with adrenergic receptor stimulation with norepinephrine and combines this with a predicted hypothetical participation of the redox-activated mitochondrial phospholipase iPLA2γ providing nascent fatty acids for UCP1-mediated uncoupling, thus substantiating heat production. Also, depicted is the possible is the participation of thioredoxin TRX3 in the transfer of redox signal resulting in sulfenylation of critical cysteine 253 for activation.
Abbreviations: β3-AR – β3- adrenergic receptor; AC – adenylated cyclase; ASCT – glutamine transporter; ATGL – adipose triglyceride lipase; αGP – α-glycerolphosphate; αGPDH – α-glycerolphosphate dehydrogenase; CD36 – fatty acid transporter; CI, CII, CIV– respiratory chain complexes; ETFQOR – electron transfer flavoprotein: uniquinone oxidoreductase; FA – fatty acid; GLUT – glucose transporter; HSL – hormone-sensitive lipase; RCoA – acylCoA; iPLA2γ – calcium-independent phospholipase A2γ; MnSOD – manganese superoxide dismutase; PKA – protein kinase A; RCarn – acylcarnitine; SDH – succinate dehydrogenase; SN2 – glutamine transporter; TG – triglyceride.

leads to heat generation and the essential thermogenic role of UCP1 for nonshivering thermogenesis in rodents has been confirmed by comparison with UCP1 knockout mice [91–94] (Fig. 3).

The norepinephrine stimulation cascade, controlling both acute thermogenesis and BAT recruitment, involves at its first step a coupling of the β-adrenergic receptor with a Gs protein which activates adenylyl cyclase leading to formation of a second messenger cAMP [95,96]. Subsequently, cAMP promotes PKA-mediated phosphorylation of cytosolic lipolytic enzymes and other proteins including nuclear proteins. The main acute response thus includes lipolysis of lipid droplets by activation of hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL), and perilipin. Lipolysis produces free FAs, which as UCP1 cycling substrates, mediate mitochondrial uncoupling and heat production. FAs are imported into the matrix by the so-called carnitine cycle whereby acyl-CoA's are condensed to acyl-carnitine esters by carnitine palmitoyltransferase 1 (CPT1). The mitochondrial carnitine carrier then imports long chain acyl-carnitine esters to the matrix in exchange for short-chain acylcarnitines or L-carnitine. Acyl carnitines are converted back to acylCoA by CPT2 and acylCoA's are substrates for β-oxidation in the matrix, producing superoxide by ETF:QOR protein feeding electrons via FAD to the respiratory chain. In the non-activated resting state, FAs in BAT are recruited into the lipid droplets by lipoprotein lipase and the FA transporter CD36, the ablation of which impairs BAT function [89]. Interestingly, a glucagon peptide-1 (GLP-1) analog is also known to activate BAT in mice [97].

The chronic effects involving UCP1 stem mostly from the transcriptional regulation of UCP1 expression [98]. A distal enhancer and proximal promoter have been recognized to act in UCP1 transcriptional regulation. Both are involved in the PKA-mediated upregulation of UCP1 expression, directly via the cAMP-responsive element binding protein (CREB) recruited to the proximal promoter, where also the PRD1-BF-1-RIZ1 homologous domain-containing protein-16 (PRDM16) binds to flanking regions [99], either with CCAAT-enhancer binding proteins α and β, or with lysine-specific demethylase and zinc finger protein 516. Alternatively, PKA via p38 mitogen-activated protein kinase (p38 MAPK) activates the activating transcription factor 2 (ATF2) in the distal enhancer. The ATF2 region is preceeded by three retinoid receptor (RXR) sites, all stimulated by peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1α) [100]. The middle site can be activated by fibrates and thiazolidinediones via peroxisome proliferator-activated receptor α or γ (PPAR α or γ), which also requires binding of PRDM16, PGC1α, receptor-interacting protein 140 and liver X receptor [98]. Thyroid hormones manifest their effect via the most distal site upon complex formation of the thyroid receptor with RXR and PGC1α.

Thus the main chronic effects of adrenergic stimulation are driven by PKA-mediated phosphorylation of nuclear proteins, which leads to BAT growth by hyperplasia and hypertrophy, increased mitochondrial biogenesis via p38 MAPK signalling and elevating PGC1α expression. Simultaneously BAT angiogenesis stems from participation of VEGF. Note also that BAT biogenesis is stimulated via the JAK/STAT pathway [101] and by Sirtuin 1 (SIRT1) [102]. Interestingly, without “alarmin”, i.e. the IL33 nuclear cytokine activating type 2 immune response, brown adipocytes develop without appropriately spliced *ucp1* mRNA and hence do not express UCP1 protein [103].

3.2. Brite/beige adipose tissue

Besides the existence of BAT, there also exists brown in white (brite) tissue (also termed beige) at certain *loci* within WAT [104,105]. Despite containing UCP1, beige adipocytes come from a different developmental lineage to brown adipocytes. Beige adipocytes develop from capillary networks in response to pro-angiogenic factors and consequently should probably be considered a different cell type [106]. The $myf5^+$ lineage (myogenic factor 5, myf5-dependent), which is also common for skeletal muscle, is determining in brown adipocytes, whereas a $myf5^-$ lineage is the precursor for beige adipocytes [99]. In the basal state, beige adipocyte exhibit low UCP1 expression, which is significantly enhanced upon hormonal stimuli linked to cAMP [104]. Therefore, some BAT depots, in humans considered previously as composed of brown adipocytes, might be depots of beige adipocytes.

An intriguing idea supported by the recent advances in BAT research assumes that in obesity, inflammation of WAT results in decreased biogenesis brite/beige adipocytes, which otherwise contribute to elevated energy expenditure within WAT, because of their functional thermogenic UCP1 [13]. The ability of overexpressed UCP1 in pig WAT (pigs don't have UCP1) to decrease fat depositions and enhancing thermogenic capacity has been suggested as proof of the concept [107]. The molecular mechanisms involved in this etiology type of obesity development are widely studied. The inhibitory mechanism for beige adipogenesis involves direct adhesion of macrophages to adipocyte integrin α4 resulting in ERK-dependent downregulation of UCP1

expression [108]. The involvement of paracrine secretion in these macrophage-WAT interactions downregulating oxidative phosphorylation related respiration in humans was also indicated [109]. Also inhibition of signalling by the Gq family of G-protein coupled receptors (GPRC) enhances differentiation of human and murine brown adipocytes [110]. The endothelin/Ednra pathway was identified as an autocrine activator of Gq signalling in BAT. Gq expression in human WAT inversely correlated with UCP1 expression, consequently Gq signalling is thought to negatively regulate brown/beige adipocyte biogenesis.

Recent advances in UCP1 expression include the discovery of the aforementioned alarmin/IL-33, an essential factor, the absence of which results in beige and brown adipocytes not expressing an appropriately spliced *ucp1* mRNA [103]. Beige adipocytes exhibit inducible UCP1 expression in response to cold exposure [111,112]. Their adipogenesis stems, on the one hand from committed progenitor cells and on the other hand, from the transdifferentiation of white adipocytes. The latter process is believed to be stimulated by type 2 cytokine signalling of macrophages [113].

Despite all the aforementioned, two types of beige adipocytes have been proclaimed to found, those that express UCP1 and those that do not express UCP1 [114]. Both types have been implicated with another mechanism of molecular heat production base on the creatine dissipative cycle. In the UCP1 positive beige adipocytes the UCP1-related heat production provided the additional heat source. Interestingly, UCP1 was detected in neurons of thirteen-lined ground squirrels, the proposed role there being brain thermogenesis during hibernation [115].

3.3. BAT in humans

Existence of BAT in newborn humans has long been known [116,117]. Functional BAT has been recognized also in adult humans in cervical, supraclavicular, paravertebral, mediastinal, and perirenal regions [118–121]. Interestingly, the haplotypes covering the *ucp1* region correlating with higher non-shivering thermogenesis score also correlated with more northern latitude, showing that during evolution UCP1 gene was related to cold adaptation [122].

PET studies using ^{18}F-fluoro-deoxyglucose (FDG) have confirmed the existence of functional BAT in adult humans. Adults retain a minor amount of BAT past infancy (30 to 85 g in "high BAT subjects contrasting to < 2 g BAT for low BAT" subjects) but that BAT contributes only a small amount to cold-induced thermogenesis [123]. That amount was quantified recently using determinations of human BAT oxygen consumption by $[^{15}O]O_2$ PET imaging during cold exposure of human subjects. The results indicated that BAT contributed only ~1% of total body elevation in energy expenditure [124]. Interestingly mitochondria of permeabilized human BAT, obtained by a PET-CT guided biopsy, showed a 50-fold respiratory capacity versus human WAT, with a significant proportion of that oxygen consumption being inhibitable by GDP in BAT [125]. BAT in humans is activated under the same conditions as in rodents and under systemic impairment of lipid metabolism [126]. It has been reported that such activation improves glucose homeostasis and insulin sensitivity in humans [127].

Brite/beige adipogenesis has also been identified in humans, e.g. under adrenergic stress [128]. The exposure of human subjects to the cold promotes secretion of FGF21 and the myokine, irisin, both of which stimulate formation of beige adipocytes [129]. Other players in brown fat activation and WAT browning include MP10, an inhibitor of phosphodiesterase 10A which stimulates BAT biogenesis, and "browning" of WAT [130] and MKK6, an activator of p38 MAPK, ablation of which increases UCP1 expression in adipocytes and T3-dependent browning of white adipocytes [131].

4. Thermogenic function only within the promoting environment

4.1. Heat promoting aspects in BAT

In terms of heat production, microcalometric studies indicate that BAT in cold-acclimated rats has five times the power (mW) of BAT in non-cold acclimated animals [28]. The heat released from palmitoylcarnitine oxidation by BAT mitochondria clearly shows a reciprocal relationship between Δp and heat release [29]. Furthermore, intact isolated brown adipocytes were demonstrated to produce heat when stimulated with norepinephrine [28]. Likewise significant temperature increases (by 1 °C) have been reported in the media of a liquid culture calorimeter when yeast strains expressing endogenously active UCP1 mutants were grown [132]. Moreover, temperatures of up to 50 °C has been reported in HEK cells expressing UCP1 [133]. Nevertheless the significance of the fraction by which UCP1-mediated heat release contributes to whole body non-shivering thermogenesis has been debated over the decades and, as mentioned above, the fraction of heat release specifically in adult humans, is rather low.

4.2. Switching between mild and thermogenic uncoupling

In intact brown adipocytes UCP1 is constantly inhibited by purine nucleotides (ATP, ADP, GTP or GDP) penetrating into the mitochondrial ICS from the cytosol. The purine nucleotide inhibition is overcome upon norepinephrine stimulation. The mechanism by which purine nucleotide inhibition of UCP1 occurs is not yet fully elucidated. However, it is plausible that conformational changes induced by fatty acids are involved. For example, the binding of GDP to UCP2 induces conformational changes affecting Gly281 and Gly19 [52]. Pioneering electron spin resonance (EPR) studies indicated that 5-doxyl-C18 FA interactions with UCP1 were affected by GDP-induced conformational changes [26,134]. The "open" and "closed" state of UCP1 has been simulated using isolated BAT mitochondria under physiological Mg^{2+} conditions and using physiological ATP, ADP and AMP concentrations found in nonepinephrine-stimulated and resting state BAT, respectively [25]. Since it has long been known that alkaline pH decreases purine nucleotide inhibition of UCP1 [24,135,136], one might predict a physiological role for the local ICS pH becoming more alkaline upon uncoupling, however indications are that in uncoupled mitochondria ICS pH is rather acidic especially at cristae edges containing ATP-synthase dimers [83].

It has long been postulated that mild uncoupling, a partial dissipation of Δp, is involved in regulating mitochondrial superoxide formation [1]. However, switching between mild and thermogenic uncoupling does not consist only of switching on UCP1 while insuring its persistent expression (one also sees suppression of ATP-synthase expression in BAT), but thermogenic uncoupling requires a continuous supply of respiratory chain substrates. For instance, uncoupled respiration requires β-oxidation of FAs not only provided by norepinephrine-stimulated lipolysis of lipid droplets (and α-glycerol phosphate from those sources), but also fatty acids from lipoproteins developed as physiological means of prolongated non-shivering thermogenesis [11].

Resting brown adipocytes, i.e. non-thermogenic brown adipocytes are also interesting metabolically. For example, brown adipocyte cell lines exhibit reductive carboxylation, i.e. isocitrate dehydrogenase 2 (IDH2)-mediated counter Krebs cycle directed substrate flux [137]. In this case, glutaminolysis provides 2-oxoglutarate which is channelled at the expense of NADPH into isocitrate, and via cis-aconitate to citrate, which is exported from the matrix to the cytosol for lipogenesis. It is not known how active this reductive carboxylation is upon norepinephrine stimulation.

4.3. UCP1 function in the absence of an environment promoting thermogenesis

A non-thermogenic but antioxidant function for UCP1 has been found in a diverse group of mammals [138]. Due to the uncoupling nature of UCP1, one might expect lowered ROS production in mitochondria containing active UCP1. There is certainly evidence from isolated mitochondrial studies that UCP 1 can reduce ROS production by BAT mitochondria [126,127] and thymus mitochondria [139], using a variety of mitochondrial substrates. However, others suggest that any role UCP1 may have in regulating ROS production in BAT mitochondria may not be a general rule for all substrates [140]. However, there is evidence emerging, discussed in the next section, of a role for ROS in regulating UCP1 activity.

4.4. Redox regulation of UCP1 function

Physiologically triggered covalent modification of UCP1 may not seem too radical an idea, but it is only in recent years that covalent modifications of UCP1 have been detected. The proportion of UCP1 that is phosphorylated (Ser 51) is certainly increased following cold acclimation, yet the role of this covalent modification is unclear [141]. It has also been demonstrated that UCP1 turnover in both BAT and thymus mitochondria requires the proteasome- ubiquitinylation system [142]. Recently, it was revealed that postranslational modifications can provide a switch that initiates nonshivering thermogenesis in BAT [36,66]. All three species of ROS, namely mitochondrial superoxide, hydrogen peroxide, as well as lipid hydroperoxides, were indicated in this mechanism upon cold exposure of mice. Mitochondrial targeted antioxidant MitoQ prevented these changes, which were also absent in $UCP1^{-/-}$ mice. MitoQ also induced hypothermia upon cold exposure but did not affect muscle shivering which would be considered an early thermogenic response to cold. It was also demonstrated that upon cold exposure, the BAT glutathione pool was extensively reduced and BAT protein thiols, including sulfenyls, were extensively oxidized. An instant rise in superoxide was also observed in isolated brown adipocytes upon norepinephrine stimulation, typically associated with increased (uncoupled) respiration. It transpires that Cys253 of UCP1 is sulfenylated under noradrenaline stimulation. Interestingly, Cys253 is conserved in UCP2, UCP3, and faces the matrix side on structural models of UCP1, based on the crystal structure of the ADP/ATP carrier structure. Replacement by Cys 253 with Ala (as well as replacement of Cys224) resulted in blockage of norepinephrine-induced respiratory rise, i.e. uncoupling and increased sensitivity to GDP inhibition. It should also be noted that in the case of the Cys253Ala UCP1 mutant (but not for Cys224Ala), norepinephrine, at higher doses, overcame the aforementioned insufficiency.

4.5. When a rise of mitochondrial ROS sources overwhelms their attenuation by uncoupling

Uncoupling even in its mildest form (a few mV reduction in Δp) possesses the ability to attenuate superoxide formation originating from certain mitochondrial sources (those dependent on Δp), specifically from Complex I and Complex III Q sites (for detailed review see [55,83,143–145]. Evidence has also been presented of direct activation of UCP1 by certain ROS generated products such as 4-hydroxy-nonenal (4-HNE) modification, facts that were disputed by later studies [146–148]. Indeed, it has been shown that lipid ethanolamine modifications, by reactive aldehydes such as 4-HNE, can cause increased proton leak [149]. Nevertheless, the hypothesis of a ROS-induced attenuation of superoxide formation has been made plausible by the observation of UCP1 Cys253 sulfenylation. Nevertheless, consequences of this redox UCP1 modification on acute or chronic ROS formation is still an area of robust investigation.

Cold-induced stimulation of BAT when UCP1 is ablated, promotes excessive mitochondrial ROS production that, contrary to the situation in wild-type mice, induces mitochondrial Ca^{2+} overload and concomitant dysfunction, as indicated by markers of cell death and recruitment of innate immune signalling [150]. The impaired mitochondrial function of UCP1 KO mice exhibited lower amounts of respiratory chain subunits encoded by mtDNA, and decreased gene expression for oxidative phosphorylation [150].

4.6. Non-canonical roles for UCP1

Apart from being in BAT and beige/brite adipose tissue, there are layers of convincing evidence that UCP1 is present in thymus [152,153], although there is some evidence to the contrary [151]. The insensitivity of UCP1 expression in thymocytes to cold-adaptation suggests no thermogenic role for UCP1 in thymus [152]. However, an absence of mitochondrial UCP1 affects apoptosis in thymocytes, resulting in reduced peripheral (spleen) T-cell production by up to two-thirds, and an overall reduced (~85%) in CD8 + T-cells production [153].

Transparency document

The Transparency document associated with this article can be found, in online version.

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