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Mitochondrial Ca^{2+}, the secret behind the function of uncoupling proteins 2 and 3?

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Summary

The underlying molecular action of the novel uncoupling proteins 2 and 3 (UCP2 and UCP3) is still under debate. The proteins have been implicated in many cell functions, including the regulation of insulin secretion and regulation of reactive oxygen species (ROS) generation. These effects have mainly been explained by suggesting that the proteins establish a proton leak through the inner mitochondrial membrane (IMM). However, accumulating data question this mechanism and suggest that UCP2 and UCP3 may play other roles, including carrying free fatty acids from the matrix towards the intermembrane space, or contributing to the mitochondrial Ca^{2+} uniport. Accordingly, in this review we reflect on these actions of UCP2/UCP3 and discuss alternative explanations for the molecular mechanisms by which UCP2/UCP3 might contribute to aspects of cell function. Based on the potential role of UCP2/UCP3 in regulating mitochondrial Ca^{2+} uptake, we propose a scheme whereby these proteins integrate Ca^{2+}-dependent signal transduction and energy metabolism in order to meet the energy demand of the cell for its continuous response, adaptation, and stimulation to environmental input.

Keywords

Ca^{2+} signaling; Diabetes; Fatty acid transport; Glucotoxicity; Lipid metabolism; Lipotoxicity; Mitochondria; Mitochondrial Ca^{2+} uniporter; Insulin secretion; Uncoupling proteins; UCP2; UCP3; ROS

Introduction

The original uncoupling protein, UCP1 (Link to HPRD), also called thermogenin, was first described in 1976 and was found to be almost exclusively expressed in the brown adipose tissue of mammals, where it is responsible for the inducible thermogenic activity of this tissue (for review see ref. [1]). UCP1 accounts for a proton (H^+) influx through the inner mitochondrial membrane (IMM) along the H^+ gradient that is established by the mitochondrial respiratory chain and thus, uncouples mitochondrial respiration from ATP production (ATP-synthase). Noteworthy, brown adipose tissue was found to contain very

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low amounts of ATP-synthase, compared to the components of the respiratory chain [2], thus, UCP1, which accounts for 10% of the protein mass in the IMM [1], is mainly responsible for the dissipation of the proton gradient at the IMM in this particular tissue. 21 years later, three groups found from databases of expressed sequence tags or from cDNA libraries two mRNAs encoding for two proteins that share 72% homology with each other and approximately 58% with UCP1 [3—5]. Based on this existing sequence homology with UCP1, the proteins were named uncoupling protein 2 (UCP2) and uncoupling protein 3 (UCP3) although no function could be attributed to them at this time. Meanwhile, for UCP3 two splice variants $UCP3_L$ (for long) and $UCP3_S$ (for short) were identified of which $UCP3_S$ lacks the sixth potential transmembrane region and the purine nucleotide binding domain [5].

In contrast to UCP1, the novel uncoupling proteins UCP2 and UCP3 were found in tiny concentrations of 0.01—0.1% of the mitochondrial membrane proteins in numerous tissues (UCP2: Link to HPRD; UCP3: Link to HPRD) [6]. Since both proteins are also expressed in ectothermic fish and plants that do not require thermogenesis an exclusive thermogenic function of UCP2 and UCP3 seemed unlikely and other molecular actions of UCP2 and UCP3 were sought. Following the original concept, UCP2 and UCP3 have been extensively studied in isolated systems in terms of their uncoupling potential. However, while these studies often report considerable uncoupling potential of these proteins, at least under certain conditions, controversial findings were obtained in intact cells. In this respect, Nedergaard and Cannon suggested that while the naming of UCP2 and UCP3 is comprehensible, considering their similarity to UCP1, in view of the accumulated experimental evidence that indicate that UCP2 and UCP3 most likely do not act as uncoupling proteins, this nomenclature might be misleading and may "*direct thoughts towards the implied function*" [7]. Indeed, despite experimental evidence of alternative functions of UCP2 and UCP3 other than uncoupling by forming a H^+ channel, many reports that deal with these proteins discuss the contribution of UCP2 and UCP3 entirely on basis of their uncoupling functions. Very recently we made some novel and totally unexpected observations that suggested alternative (or additional) functions for UCP2 and UCP3. Using overexpression, knock-down techniques and $UCP2^{-/-}$ animals we found that both proteins are required for mitochondrial Ca^{2+} sequestration through the classical mitochondrial Ca^{2+} uniport [8]. Most of this work was performed in intact cells following stimulation with agonists which trigger an increase of the cytosolic Ca^{2+} concentration. Although it remains to be proved whether or not this Ca^{2+} function of UCP2 and UCP3 is the main physiological task of these proteins, these findings question the physiological role of UCP2 and UCP3.

Consequently, in this review, we challenge the uncoupling function of UCP2 and UCP3 and attempt to provide alternative explanations for published phenomena that describe the role of these proteins in mitochondrial Ca^{2+} signaling.

Molecular functions of UCP2/UCP3

At first glance, there are at least three independent molecular actions reported for UCP2 and UCP3: (i) establishing a proton leak through the IMM, (ii) carrying free fatty acids from the

matrix to the intermembrane space, and (iii) contributing to mitochondrial Ca^{2+} uniporter activity.

Establishing a proton leak

According to the chemiosmotic theory defined by Peter Mitchell, the oxidation of the reduced coenzymes $NADH/H^+$ and $FADH_2$ at complexes of the respiratory chain at the IMM is linked to the transport of protons from the matrix to the intermembrane space [9]. Thus, the electron transfer to molecular oxygen by protein complexes I—IV leads to an electrochemical gradient that establishes a negative membrane potential ($\Delta\psi_{mito}$) of the IMM at around −180 mV. At this potential, protons flow back into the mitochondrial matrix via the F0-F1-ATPase and the released energy is utilized to phosphorylate ADP to ATP [10]. Notably, $\Delta\psi_{mito}$ is of utmost significance for most mitochondrial (bioenergetic) functions and constitutes the driving force for the influx of cations such as H^+ and Ca^{2+} into these organelles (for reviews see refs. [11,12]). Since a total and continuing loss of $\Delta\psi_{mito}$ results inescapably in cell death, $\Delta\psi_{mito}$ is maintained by multiple processes (including the respiratory chain) that compensate any kind of disturbances.

Once a high $\Delta\psi_{mito}$ is achieved, an additional increase of substrate supply cannot further activate respiration due to the high H^+ gradient across the IMM [13]. Basically, proton influx through the ATP-synthase-complex counteracts the established high H^+ gradient and thus, maintains activity of the respiratory chain. Accordingly, inhibition of ATP synthesis should cause a total loss of respiration. However, it has been demonstrated that isolated mitochondria still consume O_2 even if the ATP-synthase is entirely inhibited [14], thus, indicating that the H^+ gradient must be lowered via routes others than the ATP-synthase. To date, it is not entirely clear whether such a H^+-leak across the IMM, which displays an energy dissipating process by “uncoupling” phosphorylation of ADP from respiration, is actively controlled by proteins or if the IMM is more or less passively permeable for H^+ or small molecule H^+-carriers such as free fatty acids. In this context Skulachev proposed that several proteins located at the IMM such as UCP1, the ATP/ADP translocator, the glutamate/aspartate antiporter and the dicarboxylate carrier facilitate the translocation of (fatty acid) anions (see Section ‘Free fatty acid carrier’) from the matrix to the intermembrane space [15]. Although the molecular mechanism by which the original uncoupling protein UCP1 induces heat generation by dissipation of the H^+-gradient in brown adipose tissue is still elusive, it seems plausible that fatty acids are essential for stimulating uncoupling activity of UCP1 [16]. Currently, two distinct hypotheses exist, where the first suggests that UCP1 directly functions as an inward H^+ carrier [17], whereas the other suggests that UCP1, as well as UCP2 and UCP3, the ATP/ADP translocator and the glutamate/aspartate carrier, are involved in a free fatty acid cycling mechanism [18—20] transporting free fatty acid anions from the matrix side to the intermembrane space (Fig. 1A). The H^+ conductance is facilitated by the free fatty acid themselves, which are protonated on the outer leaflet of the inner membrane and pass through the membrane by a “flip-flop” mechanism, releasing protons in the mitochondrial matrix.

It has been calculated that the H^+-leak in mammalian liver and skeletal muscle accounts for approximately 20% of basal O_2 consumption [21]. If uncoupling is considered as a

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controlled physiological process, it can be assumed that other proteins in the IMM are involved in this process in all tissues lacking UCP1. The two UCP1 homologues, UCP2 and UCP3 are expressed in a variety of tissues [3—5]. Notwithstanding, a strong uncoupling function of these novel UCPs under physiological conditions could not be demonstrated in intact cells so far. This is partially due to the fact that an excessive, unphysiological overexpression of any (misfolded) protein located at the IMM obviously has the potency to enhance respiration by increasing the H^+-leak across the IMM artificially [22,23]. Hence extensive research on the molecular function of UCP2 and UCP3 performed in recent years, including data from knock-down approaches, remain controversial in terms of the participation of UCP2 and UCP3 in uncoupling and associated thermogenesis. Indeed, several reports exclude a typical uncoupling function of UCP2 or UCP3 [24—26]. On the other hand, a reduced H^+-leak and increased ATP levels at least in some tissues of UCP2 or UCP3 ablated mice have been observed, consistent with suggestions that these proteins facilitate uncoupling of respiring mitochondria [27—29]. Notably, there is some evidence that UCP2 and UCP3 accomplish mild uncoupling, which is a term that implies a small increase in H^+-leak associated with a slight reduction of the H^+-gradient with little or no effect on ATP production [30]. Moreover, it has been suggested that mild uncoupling is activated by ROS and reactive alkenals such as 4-hydroxynonenal and represents a negative feedback to mitigate mitochondrial ROS generation (see Section ‘Defense of mitochondrial ROS’) [31].

Mitochondrial uncoupling, the dissipation of the H^+-gradient of the IMM resulting in enhanced respiration not coupled to chemical or osmotic work but associated with heat production, can be scrutinized adequately only under rather unphysiological conditions using suspended isolated mitochondria [32] or liposomes [33] and in the presence of certain substrates and toxins. Accordingly the contribution of UCP2 and UCP3 to mitochondrial uncoupling in intact cells or tissues, and the relevance of this process for physiology and pathophysiology remains unclear and requires further investigations.

Free fatty acid carrier

Uncoupling proteins are inhibited by purine nucleotides and activated by free fatty acids (for review see ref. [34]). The interaction of fatty acids with UCP1 is very well characterized in mitochondria from brown adipose tissue, but was also shown for members of the mitochondrial anion carrier protein family, e.g. ANT or the glutamate/aspartate carrier (see review ref. [15]). These proteins are considered to mediate a purine nucleotide-sensitive uniport of monovalent unipolar anions, including anionic fatty acids. The transport of protonated fatty acids from the intermembrane space to the mitochondrial matrix and the subsequent release of the H^+ from the polar fatty acid headgroup leads to net H^+ uniport and uncoupling [18,35,36]. Moreover, the export of excessive free fatty acids from the mitochondrial matrix by UCP2/UCP3 has been suggested to rescue mitochondria from an overload of free fatty acids (the ‘rescue hypothesis’ of Schrauwen [37—39]) (Fig. 1A).

Mitochondrial Ca^{2+} uptake

The contribution of mitochondria to cellular signaling cascades as well as most of the organelle’s own functions are strikingly linked to mitochondrial Ca^{2+} (for review see ref.

[12]). Mitochondrial Ca^{2+} homeostasis is concertedly accomplished by multiple Ca^{2+} channels, pumps and exchangers which, remarkably, mostly remain genetically unidentified. We have recently demonstrated that UCP2 and UCP3 are fundamentally involved in mitochondrial Ca^{2+} uniport (MCU; [8]), the process that represents the main Ca^{2+} sequestration pathway into the mitochondrial matrix [40,41] (for review see ref. [12]). This conclusion is based on the following experimental findings (Table 1):

- **i.** siRNA-triggered knock-down of UCP2/UCP3 abolishes mitochondrial Ca^{2+} accumulation in response to cytosolic Ca^{2+} elevation/Ca^{2+} entry in intact cells.
- **ii.** Overexpression of either UCP2 or UCP3 was associated with enhanced mitochondrial Ca^{2+} accumulation in response to cell stimulation.
- **iii.** Isolated liver mitochondria of $UCP2^{-/-}$ mice lacked ruthenium red-sensitive mitochondrial Ca^{2+} uptake, while it was present in the liver mitochondria of wild type littermates that contain UCP2 mRNA and protein [8] (Fig. 1A). Notably, in isolated liver mitochondria of $UCP2^{-/-}$ mice, a ruthenium red-insensitive mitochondrial Ca^{2+} uptake that exhibited approximately 50% of that found in liver mitochondria of wild type littermates was found. Whether this phenomenon represents an adaptation of the tissue in response to the lack of UCP2 or is the reason for the lack of a strong phenotype of the knock-out mice is unclear.
- **iv.** Site-directed mutagenesis of UCP2 and UCP3 revealed the formation of dominant negative proteins that competitively counteracted the Ca^{2+} function of the wild type proteins.

Consistent with these findings, UCP2 and UCP3 were described to be activated by fatty acids and blocked by nucleotides [36,42,43], a phenomenon that was also recently reported for the ruthenium red-sensitive mitochondrial Ca^{2+} uniporter [44,45].

The findings on the contribution of UCP2/UCP3 to mitochondrial Ca^{2+} uptake stand against the dogma that UCP2/UCP3 depolarize mitochondria as this effect should reduce the electrical driving force for Ca^{2+} accumulation, and thus on would expect mitochondrial Ca^{2+} uptake to be decreased if UCPs are overexpressed. In contrast to UCP2 and UCP3, overexpression of UCP4 reduced store-operated mitochondrial Ca^{2+} influx in PC12 cells [46]. Although neither mitochondrial depolarization nor uncoupling activity of UCP4 in the intact PC12 cells was measured so far, the molecular action of UCP4 that underlies its inhibitory potential on store-operated Ca^{2+} influx in PC12 cells may point to an uncoupling potential of this more recently described UCP homologue [47].

The controversy on the molecular action of UCP2 and UCP3 is fueled by multiple reports that describe convincingly an uncoupling activity of these proteins in isolated membranes or bilayers while such activity could not be directly measured in intact cells [19,23,34,42]. Consequently, UCP2/UCP3 have been postulated as carriers of ions other than H^+ that do not cause uncoupling, but control mitochondrial metabolic activity in CHO cells [48].

Strikingly, heterologous expression of human UCP3 in yeast did not reveal any evidence for a uncoupling activity of this protein and lead the authors to conclude “that the uncoupling of yeast mitochondria by high levels of UCP3 expression is entirely an artifact and provides no

evidence for any native uncoupling activity of the protein" [23]. However, the same yeast models for heterologous expression of either UCP2 or UCP3 exhibited a strong ruthenium red-insensitive mitochondrial Ca^{2+} accumulation upon extramitochondrial Ca^{2+} while the heterologous expression of UCP2/UCP3 failed to establish ruthenium red-sensitive mitochondrial Ca^{2+} uniport in these yeast models [8]. Accordingly, expression of UCP2/UCP3 in yeast could not provide any support for either uncoupling or Ca^{2+} function of these UCPs. Whether yeast, which does not express an UCP homologue, lacks further proteins that are essential to establish either the ruthenium red-sensitive mitochondrial Ca^{2+} uniporter or H^+ conductance, or the molecular action of UCP2/UCP3 are distinct from these postulated activities is unclear and needs further attention.

Overall, the contribution of UCP2 and UCP3 to mitochondrial Ca^{2+} uptake (Fig. 1A) brings some light to the understanding of the molecular action of UCP2 and UCP3 while additional studies are necessary to further approve the "Ca^{2+} function" of these proteins. However, in view of the recent data and the ongoing uncertainties whether or not UCP2 and UCP3 uncouple mitochondria in intact cells (see Section 'Establishing a proton leak'), their effect on mitochondrial Ca^{2+} signaling needs to be considered in the attempt to explain the underlying molecular mechanisms of the physiological effects of UCP2 and UCP3.

Does (UCP2/UCP3-mediated) mitochondrial Ca^{2+} flux mimic uncoupling?

In view of the increasing evidence that UCP2 and UCP3 do not exhibit uncoupling function under physiological conditions, the nomenclature of these two proteins, which originally stems from their similarities with UCP1, was claimed to be inappropriate by several authors (e.g. ref. [7]). However, the recent findings on the contribution of UCP2 and UCP3 to mitochondrial Ca^{2+} uptake provides a mechanistic scenario in which these proteins indeed should yield (mild) uncoupling as functional consequence of mitochondrial Ca^{2+} accumulation. In most cell types, the extrusion of elevated mitochondrial Ca^{2+} is achieved by the mitochondrial Na^+/Ca^{2+} exchanger (NCX_{mito}; [49,50]). Subsequently, Na^+ is extruded by a, so far unidentified Na^+/H^+ exchanger, which causes mitochondrial acidification, diminishing the H^+ gradient across the IMM [12] (Figs. 1B and 2). Accordingly, Ca^{2+}-dependent acidification of the mitochondrial matrix increases the activity of the respiratory chain without increasing ATP formation, as the reduction of the H^+ gradient reduces the activity of the ATP-synthase. Since, by definition an increase in the activity of the respiratory chain that does not result in "useful work", such as enhanced ATP formation is referred as uncoupling [15], Thus, the Ca^{2+} uptake function of UCP2/UCP3 may indeed yield mitochondrial uncoupling although these proteins may not exhibit a H^+ conductance directly.

Notably, such Ca^{2+}-mediated mitochondrial uncoupling counteracts the stimulatory effect of Ca^{2+} on the activity of the mitochondrial dehydrogenases [51—55]. The outcome of this bidirectional regulation of mitochondrial ATP formation by Ca^{2+} might depend on the amount of Ca^{2+} sequestered by the mitochondria and may be stimulatory at lower and inhibitory at (pathologically) high intraluminal Ca^{2+} concentrations (see Section 'UCP2 and islet function').

Physiological functions of UCP2/UCP3 and their implication in pathology

Although the molecular mechanism(s) of UCP2 and UCP3 is/are still under debate there are some identified physiological functions that point to the importance of UCP2 and UCP3 in human physiology and pathology. As the physiological functions of UCP2 and UCP3 have been extensively reviewed recently [7,19,42,56—66], herein we selected the most prominent functions of these proteins and reconsider the suggested explanation in view of the Ca^{2+} function of UCP2 and UCP3: (i) islet function, (ii) defence of mitochondrial ROS production, and (iii) free fatty acid metabolism.

UCP2 and islet function

The oxidative metabolism of D-glucose and mitochondrial ATP production represent the key phenomenon that couples D-glucose concentration with pancreatic insulin secretion, a process referred as glucose-stimulated insulin secretion (GSIS) (for review see ref. [67]). Thus, D-glucose-induced mitochondrial ATP production that results in an elevation of cytoplasmic ATP/ADP ratio triggers insulin secretion both by blocking K_{ATP}-dependent channels and by K_{ATP}-independent mechanisms [67,68]. In view of the regulatory role of mitochondrial ATP production for GSIS, any mechanism that reduces the efficiency of mitochondrial ATP production at given activity of the respiratory chain (i.e. uncoupling) will inevitably impair insulin secretion. Since, UCP2 is normally expressed in both human and rodent pancreatic beta cells[69,70], a role of UCP2 as a negative regulator for pancreatic insulin section was proposed [70].

Evidence—An inhibitory role of UCP2 for GSIS has been described in many reports in which GSIS was reduced under conditions of upregulated UCP2 expression [69—71], while the absence of UCP2 enhances GSIS [28,72]. Moreover, an upregulation in UCP2 expression that was associated with signs of mitochondrial uncoupling was described after long term exposure of beta cells to high concentrations of fatty acids [73—75] but not by nutrition oversupply (i.e. fatty acids plus high D-glucose) or high D-glucose alone [74]). On the other hand, $UCP2^{-/-}$ mice have increased circulating insulin levels and isolated pancreatic islets from these mice secrete more insulin and show a higher ATP/ADP ratio in response to D-glucose [28]. Consistently, viral expression of UCP2 reversed these effects in beta cells isolated from $UCP2^{-/-}$ mice [76].

The controversy in explaining the molecular mechanisms of UCP2 in its contribution to GSIS—Most of these reports fuel the hypothesis that UCP2-mediated uncoupling reduces mitochondrial ATP production. As a consequence of the reduced cytosolic ATP/ADP ratio, K_{ATP} channels are insufficiently inhibited and thus, membrane depolarization is inadequate to trigger Ca^{2+} influx through voltage gated Ca^{2+} channels, which is essential for ample insulin secretion [63,68]. This concept was recently revisited as superoxide anions were found to activate UCP2 and to enhance their mitochondrial depolarizing activity[30,77,78]. Since superoxide anions are generated under overflow conditions by the respiratory chain, it was suggested that metabolism-triggered ROS production under conditions of long term high fatty acid exposure might be responsible for

beta cell dysfunction and the inhibitory action of UCP2 on GSIS (for review see refs. [42,63,79,80]).

The concept was further elaborated by the introduction of free fatty acids into the model (see Section 'Free fatty acid metabolism'). In a recent study using planar lipid bilayers, UCP1 and UCP2 were found to be activated by polyunsaturated fatty acids while saturated fatty acids had much less effect [81]. In view of the potential of fatty acids to initiate UCP2-dependent decrease in GSIS as well as upregulation of UCP2 expression [82,83], these findings further add to the idea that under metabolic overload, increased mitochondrial ROS production and fatty acid content stimulate UCP2, leading to uncoupling and reduced ATP production, eventually causing reduced GSIS[42,68,80].

While these findings are consistent with an uncoupling function of UCP2, not all studies that dealt with the effect of an overexpression of UCP2 in pancreatic beta cells revealed evidence for an UCP2-triggered uncoupling that results in a decrease in GSIS [84,85]. In transgenic mice with pancreaticspecific overexpression of UCP2 and in a respective beta cell model, D-glucose-induced ATP production and GSIS were comparable with that of control animals [84]. Moreover, no differences in resting and D-glucose-stimulated changes in $\Delta\psi_{mito}$ were found in beta cells of UCP2 overexpressing vs. control mice, thus, indicating that an UCP2 overexpression *per se* might not cause uncoupling either *in vivo* or *in situ* [84].

Thus, the findings regarding the molecular action of UCP2 in GSIS drawn from these recent reports from pancreaticspecific UCP2 overexpressing mice as well as the respective beta cell model [84] contradict the explanation derived from experiments in $UCP2^{-/-}$ mice [28].

It is notable that the overall ablation of $UCP2^{-/-}$ in mice results in slightly elevated plasma levels of inflammatory cytokines (e.g. TNFα or IFNγ) and persistent NFκB activation [86] that has been found to enhance beta cell function and insulin secretion [87]. In line with this report, attenuation of NFκB activation reduced GSIS and the expression of proteins involved in D-glucose metabolism and insulin secretion [88]. In view of these findings, Produit-Zangaffinen et al. suggested that increased GSIS in the $UCP2^{-/-}$ mice results from the activation of beta cells by NFκB rather than through a direct effect of the UCP2 on beta cell function [84].

However, the lack of any sign of mitochondrial uncoupling and reduction of GSIS by UCP2 overexpression *per se* [84] might depend on the expression level of this protein and the molecular action of moderately overexpressed UCP2 may differ from that of a supraphysiologically expressed protein [23,42]. Nevertheless, there was a striking effect of UCP2 overexpression on cytokine-induced mitochondrial ROS production in the same model [84] that is further supported by a report of the same group in which an attenuation of cytokine-induced beta cell death by increased UCP2 was described [89].

Overall, although the involvement of UCP2 in the regulation of GSIS is clear, the molecular action(s) by which UCP2 contributes to this phenomenon is still under debate and the controversy of so far reported findings may require further inputs to be finally solved.

Can the Ca^{2+} function of UCP2/UCP3 help resolve the inconsistencies between recent reports on the molecular action of UCP2 in GSIS?

Excessive mitochondrial Ca^{2+} counteracts mitochondrial ATP availability: Ca^{2+} plays a crucial role in GSIS. Insulin secretion depends predominantly on an elevation of cytosolic free Ca^{2+} [90,91] and even follows cytosolic Ca^{2+} oscillations [90,92]. Mechanistically, elevated cytosolic Ca^{2+} raises mitochondrial [Ca^{2+}], which increases mitochondrial ATP production ([93] for review see refs. [12,80]) and in turn, enhances insulin release. The stimulatory effect of Ca^{2+} on mitochondrial ATP production is thought to be mainly mediated via stimulation of several matrix dehydrogenases (pyruvate dehydrogenase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase [51—55]; for review see refs. [12,80]). In line with these observations and recent findings that demonstrated an involvement of UCP2 (and UCP3) in mitochondrial Ca^{2+} uptake [8], a moderate elevation of mitochondrial Ca^{2+} uniporter activity as a result of UCP2 (and UCP3) overexpression yields elevated ATP production upon exposure to histamine [8] an agonist that raises [Ca^{2+}]$_c$ via production of inositol 1,4,5-trisphosphate (Fig. 3B, upper panel).

The findings on the impact of UCP2 (and UCP3) on mitochondrial Ca^{2+} and ATP production are contradictory to an uncoupling function of these proteins (see Section ‘Establishing a proton leak’). Nevertheless, excessive mitochondrial Ca^{2+} sequestration via the UCP2/UCP3-containing Ca^{2+} uniporter might represent the turning point that converts mitochondrial Ca^{2+} to an inhibitory executer that prevents mitochondrial ATP production.

Since ROS promote mitochondrial Ca^{2+} accumulation [94] and stimulate UCP2/UCP3 activity (for review see ref. [42]), excessive mitochondrial Ca^{2+} accumulation might occur under physiological conditions of elevated ROS production, which increases in response to metabolic stimulation (see Section ‘Defense of mitochondrial ROS production’). Thus, as an alternative explanation of the involvement of UCP2 in GSIS, increased substrate supply that promotes electron overflow, triggering mitochondrial ROS production, might activate a UCP2-containing mitochondrial Ca^{2+} uniporter resulting in an robust mitochondrial Ca^{2+} accumulation sufficient to reduce cellular ATP levels by (i) Ca^{2+}-stimulated ATP hydrolysis [95,96], (ii) initiation of a futile, ATP consuming mitochondrial Ca^{2+} cycling [97—99], (iii) a Ca^{2+}-dependent feedback inhibition of glycolytic flux[100] and/or, (iv) Ca^{2+}-initiated uncoupling (see Section ‘Does (UCP2/UCP3-mediated) mitochondrial Ca^{2+} flux mimic uncoupling?’) (Fig. 3A). Notably, such a switch from a stimulatory effect of mitochondrial Ca^{2+} on ATP production to an inhibitory action has been also postulated recently in a model for mitochondrial ATP production [101] (Fig. 3A). Overall, such Ca^{2+}-dependent reduction in ATP production and/or increased ATP consumption will decrease GSIS. Thus this hypothesis may provide an explanation for the molecular action of UCP2 in GSIS that is consistent with all existing data so far,

Increased mitochondrial Ca^{2+} uptake suppresses cytosolic Ca^{2+} signaling: Besides the reversed metabolic action of pathologically elevated mitochondrial Ca^{2+} concentration [102] following overexpression of UCP2 to explain reduced GSIS in the respective models, mitochondrial Ca^{2+} uptake exhibits outstanding importance for shaping cytosolic and local Ca^{2+} signaling that controls multiple cell functions including insulin secretion (for review

see refs. [12,103—105]). Intriguingly, mitochondria exhibit spatial Ca^{2+} buffering in distinct area of the cytosol. In many excitable cells including cardiac myocytes, chromaffin cells and pancreas acinar cells, subplasmalemmal mitochondria buffer Ca^{2+} entering the cell by local Ca^{2+} sequestration, a phenomenon referred to as a “mitochondrial firewall” (cited from a talk of *Martin Bootman* [106]) that delays, diminishes or shapes cytosolic Ca^{2+} signaling and its accompanied cell functions [107—109]. Accordingly, in view of the involvement of UCP2 in mitochondrial Ca^{2+} uptake, upregulation of UCP2 upon substrate overload in beta cells might result in an enhanced Ca^{2+} sequestration competence of the mitochondria. Accordingly, one might hypothesize that augmented subplasmalemmal Ca^{2+} buffering by superficial mitochondria, might diminish local cytosolic Ca^{2+}-dependent processes such like vesicle transport or insulin secretion, thus, leading to a reduction of GSIS (Fig. 3B, lower panel).

Involvement of ROS in the regulatory role of UCP2 for GSIS: Recently mitochondrial ROS were found to be required for glucose/nutrition sensing of various tissues [84,110,111]. In view of these reports and the accumulating evidence that point to an involvement of UCP2 in the regulation of mitochondrial ROS production (see Section ‘Defense of mitochondrial ROS’), one might hypothesize that the regulatory role of UCP2 on insulin secretion may be associated, at least in part, with the protein’s effect on mitochondrial ROS production.

Conclusion: While there is overwhelming data that UCP2 is involved in the regulation of GSIS, the common concept on the underlying molecular mechanisms were recently challenged by increasing evidence that point to a lack of uncoupling activity of UCP2 under physiological conditions. Recent findings on the involvement of UCP2 in mitochondrial Ca^{2+} uptake and the Janus-faced contribution of mitochondrial Ca^{2+} to ATP availability might present an alternative explanation for the observed phenomena. Additional work is necessary to resolve the controversy on the molecular actions beyond the regulation of GSIS (Fig. 3B).

Defense of mitochondrial ROS production

The mitochondrial respiratory chain represents a major source of ROS [112]. It has been calculated that under basal conditions approximately 1—2% of the electrons transported by the complexes of the respiratory chain leak to produce superoxide anions [113]. The actual site of ROS generation within the complexes of the respiratory chain is not entirely clear, but complex I and complex III have attracted most attention [114,115]. ROS are potentially harmful agents that can induce severe cell damage, which has been associated with numerous diseases including diabetes, Parkinson’s disease, Alzheimer’s disease, and even ageing [58]. However, ROS are also involved in a variety of physiological signaling processes that are not linked to the development and progression of diseases [116,117]. Thus, mitochondrial generated ROS should not only be considered as toxic by-products of cellular metabolism [118,119]. Nevertheless, mitochondria are equipped with extensive ROS-defense machinery in order to balance the permanent generation of ROS and to protect against the malignant properties of ROS [120]. While mitochondrial SOD (SOD2, MnSOD) and glutathione represent the first defense line against mitochondrial ROS production [121],

there is increasing evidence that UCP2 and UCP3 also significantly serve to attenuate mitochondrial ROS production [42,122]. Although the molecular mechanism behind this function of UCP2 and UCP3 is not entirely resolved, several explanations have been proposed.

Evidence—Strong evidence for an involvement of UCP2 and UCP3 in mitochondrial ROS defense comes from the phenotype of the respective knock-out mice. For instance, in $UCP2^{-/-}$ mice increased higher ROS markers have been described in liver[123], while pancreatic islet cells dysfunction is thought to be due to the increased ROS production in these animals [78]. In line with these findings, mice with bone morrowspecific UCP2 knock-out exhibit more oxidative stress and pronounced atherosclerosis [124]. Similar to the ablation of UCP2, UCP3 knock-out results in increased ROS generation in tissues in which it is normally expressed (e.g. skeletal muscle [29]). However, although UCPs are obviously involved in the control of mitochondrial ROS production, UCP2 and UCP3 ablated mice show neither a tendency to develop cancer nor do they have a reduced life span.

Surprisingly, only a few studies with increased levels of UCP2 or UCP3 confirm the contribution of these proteins to the suppression of mitochondrial ROS. Adenovirus-mediated overexpression of UCP2 in vascular smooth muscle cells reversed elevated ROS production in response to high D-glucose and angiotensin II [125], which led the authors to suggest that agents which increase UCP2 expression in vascular cells may prevent the development and progression of atherosclerosis. In L6 muscle cells, elevated UCP3 expression reduced mitochondrial ROS production without a measurable impact on $\Delta\psi_{mito}$ [126] while this effect was not obtained by others under similar conditions in the same cell type [127].

Explanation—UCPs *per se* do not catalyze ROS conversion to less or non-reactive compounds. Thus, the molecular function of UCP2 and UCP3 responsible for ROS mitigation seems to be accomplished by an impairment of ROS-generating processes rather than being directly involved in the decomposition of already generated ROS. It is generally believed that mitochondrial ROS generation is a function of $\Delta\psi_{mito}$ [128,129]. Even under conditions of physiological pO_2-levels a high $\Delta\psi_{mito}$, in combination with a highly reduced electron transport chain and sufficient substrate availability, favors mitochondrial ROS formation. Hence, a reduction of $\Delta\psi_{mito}$, possibly by increasing the H^+-leak, was postulated as a mechanism whereby UCP2 and UCP3 may facilitate the electron flux through the respiratory chain resulting in decreased ROS formation (see Section ‘Establishing a proton leak’). Indeed, Skulachev and co-workers showed that chemical uncouplers efficiently reduce ROS production of isolated mitochondria [15,130] while in a recent study, contradictory data were described, as mild uncoupling using low doses of protonophore did not decrease mitochondrial superoxide anion levels in cultured rat cerebellar granule [131]. In line with these experiments, uncoupling does not affect mitochondrial ROS production in synaptic mitochondria[132].

In line with UCP-triggered uncoupling that counteracts ROS production, ROS-induced formation of 4-hydroxynonenal, which induces a mild uncoupling activity by UCPs was suggested [133].

Independently of any uncoupling activity, UCP2 and UCP3 protect against ROS by facilitating the transport of superoxide and hydroperoxyl radicals across the IMM (for review see ref. [7]).

Does the Ca^{2+} function of UCP2/UCP3 affect mitochondrial ROS production?—Although numerous studies have demonstrated that an excessive mitochondrial Ca^{2+} sequestration may increase mitochondrial ROS generation and even precipitate cell death [134—137], Ca^{2+} uptake into mitochondria *per se* may dissipate $\Delta\psi_{mito}$ (see Section 'Does (UCP2/UCP3-mediated) mitochondrial Ca^{2+} flux mimic uncoupling?') and thus, counteracts ROS generation [138,139]. Indeed, mitochondrial Ca^{2+} uptake is accompanied by a long lasting luminal acidification [8] that is due to the activity of either the Na^+/Ca^{2+} exchanger followed by Na^+/H^+ exchange or the Ca^{2+}/H^+ exchanger (Fig. 2). In healthy cells, changes in $\Delta\psi_{mito}$, are most likely transient [140], thus, pointing to a compensatory activity of the respiratory chain, which is activated via Ca^{2+} under such conditions. However, independently of a change in $\Delta\psi_{mito}$, the reduced proton gradient through the IMM due to the accumulation of luminal protons is by definition 'uncoupling' and may also counteract ROS production.

As long as mitochondrial ATP production is ensured even under conditions of Ca^{2+}-dependent uncoupling, the cell should behave as normal, while under conditions of decreased cellular ATP availability, the system may switch to enter the apoptosis/necrosis cell death pathways (see Section 'Excessive mitochondrial Ca^{2+} counteracts mitochondrial ATP availability').

In addition to Ca^{2+}-triggered uncoupling, mitochondrial Ca^{2+} can modulate the activity of various enzymes of the antioxidant defense system and thus, counteract ROS production [141,142].

Conclusion: A correlation of UCP2/UCP3-dependent enhanced mitochondrial Ca^{2+} influx with mitochondrial ROS production has yet to be elucidated. However, in view of existing data regarding the effect of mitochondrial Ca^{2+} on pH and enzyme activity, the roles of UCP2 and UCP3 in regulating mitochondrial Ca^{2+} may provide mechanisms for the organization and capacity of mitochondrial antioxidant defences against excessive ROS production.

Free fatty acid metabolism

The ability of UCP2 and UCP3 to transport free fatty acid anions and their participation to the mitochondrial Ca^{2+} uniport [8] fit very well to the hypothesis that these proteins are involved in the regulation of free fatty acid metabolism. A role of UCP2/UCP3 in the mitochondrial export of excessive free fatty acids that are formed by matrix thioesterase, converting acyl-CoA to free fatty acid and CoA-SH has been postulated [143]. Alternatively, free fatty acids may physically enter the mitochondria as neutral protonated fatty acids by a so-called "flip-flop" mechanism [37—39]. This is associated with a simultaneous H^+ uniport once UCP2/UCP3 export the fatty acid anions [35] (Fig. 4A). These may re-enter the β-oxidation cycle by being converted into acylcarnitine in the cytosol

and are subsequently transported into the mitochondria through the carnitine palmitoyltransferase system.

Evidence—Since UCP2/UCP3 gene expression is under the control of PPARs (peroxisome proliferator-activated receptors; for review see ref. [144]), and PPARs are important in the regulation of fat metabolism, it was suggested that UCP2 and UCP3 may play a role in fatty acid metabolism. Indeed, the expression of these proteins is upregulated under conditions of high fatty acid availability, such as obesity [145], response to fasting [57,145,146], high-fat diet [147], or acute exercise [148,149]. Moreover, UCP3 overexpressing mice showed increased fatty acid oxidation rates and decreased intramuscular triglyceride stores [146]. However, inconsistent results have been described in $UCP3^{-/-}$ mice, in which no effect on the muscular capacity to oxidize fatty acids was found[29,150].

Explanation—As outlined above evidence accumulated that point to a role of UCP2 and UCP3 in fatty acid metabolism by increasing fatty acid oxidation capacity. Notably, UCPs are more likely to transport fatty acid anions out of the mitochondrial matrix than directly translocating H^+ and thus, might work as fatty acid anion transporters (for reviews see refs. [20,36]). Consistently, UCP3 was hypothesized to represent a rescue pathway for free fatty acids when mitochondria are facing excessive free fatty acid supply [143]. Under such conditions long-chain fatty acid (LCFA)-CoA accumulate in the mitochondrial matrix, decreasing availability of coenzyme A (CoA-SH), that, in turn, could prevent further oxidation of fatty acids and limit TCA cycle activity. Hence, UCP2/UCP3 may concertedly work with the mitochondrial thioesterase in the liberation of free fatty acid anions in the matrix. The free fatty acid anions are then exported by UCP2/UCP3 and reactivated by acyl-CoA synthase in the intermembrane space. Export of fatty acid anions from the matrix by UCP2/UCP3 would not only allow greater rates of fatty acid oxidation but may also reduce $\Delta\psi_{mito}$ and thereby diminish ROS production.

Alternatively, free fatty acids that passively cross the IMM once they get protonated might be eliminated by UCP2/UCP3 as anions and thus protect mitochondria against lipid-induced damage (lipotoxicity) [37—39].

Does the role of UCP2/UCP3 in mitochondrial Ca^{2+} uptake fit with their involvement in free fatty acid metabolism?—The PPAR-dependent expression of UCP2/UCP3 may point to a coherence of the molecular function of these proteins and fatty acid metabolism. Long before the discovery of UCP2 and UCP3, it was reported that mitochondria from brown adipose tissue and liver in obese ob/ob mice have enhanced Ca^{2+} transport capacities while $\Delta\psi_{mito}$ was unaffected [151]. In view of the stimulatory effect of matrix Ca^{2+} on the rate-limiting dehydrogenases (for review see ref. [52]), mitochondrial Ca^{2+} may increase the rate of fatty acid β-oxidation in the mitochondria.

Mitochondrial fatty acid anion export and Ca^{2+} import represent two intriguing putative molecular functions of UCP2 and UCP3. This aspect generates several additional questions and hypotheses that may deserve further investigations: is there a direct link between these two proposed functions? Does the activity of the UCP2/UCP3-dependent mitochondrial Ca^{2+} uniporter depend on the presence of free fatty acids? Since numerous studies have

shown that free fatty acids affect cellular functions by modulating the activities of various ion channels and transporters, including Ca^{2+} , Cl^{-} , K^{+} , and Na^{+} , as well as non-selective cation channels [152—156], the questions raised above seem feasible. Free fatty acids may directly bind to ion channels regulating their activity or modulate channel activities through metabolites and fatty acid-activated kinases [157]. The ability of fatty acids to affect ion channels depends on the level of unsaturation and chain length. This has also been strikingly demonstrated for the mitochondrial Ca^{2+} uniporter, which is activated exclusively by polyunsaturated fatty acids [44]. In line with these findings, the total membrane anion conductance of UCP2 increased in the range “palmitic < oleic < eicosatrienoic < linoleic < retinoic < arachidonic acids” [81].

Besides the proposed concerted action of free fatty acids and UCP2/UCP3 on IMM anion conductance and mitochondrial Ca^{2+} uniport, mitochondrial Ca^{2+} and free fatty acids have been shown to activate the formation of the classical [158,159] and the so called non-classical [160,161] mitochondrial transition pore.

Conclusion: Evidence has accumulated to suggest that free fatty acids and Ca^{2+}, in a concerted action, are participating in channel/pore formation in the IMM. Nevertheless, whether the two proposed functions of UCP2/UCP3, namely the fatty acid binding/transport and the contribution to mitochondrial Ca^{2+} sequestration, are interrelated phenomena or occur independently of each other awaits further investigation (Fig. 4A).

Conclusions

Although convincing experimental data point to an involvement of UCP2 and UCP3 in multiple physiological processes, the molecular mechanisms of action of these two proteins are still hotly debated. In particular, the most common concept of a direct uncoupling activity by carrying H^{+} into the mitochondrial matrix has not been convincingly demonstrated under physiological conditions. Three striking new functions of UCPs have recently been reported and indicate involvement of UCP2 and UCP3 in mitochondrial fatty acid transport, attenuation of mitochondrial ROS production and mitochondrial Ca^{2+} uniport. So far no adequate mechanism has been described that integrates all these functions to explain the physiological role of UCP2 and UCP3. In the present review, we have discussed the most recently described function of UCP2 and UCP3 in mitochondrial Ca^{2+} uptake in the light of a range of reports that describe the involvement of UCPs in major physiological phenomena. While many questions remain unresolved, it is evident that UCP2 and UCP3 play a central role in the integration of Ca^{2+}-sensitive signal transduction, free fatty acid and substrate metabolism that are required to meet the energy demands of the cell as it responds and adapts to continuously changing energy demands (Fig. 4B).

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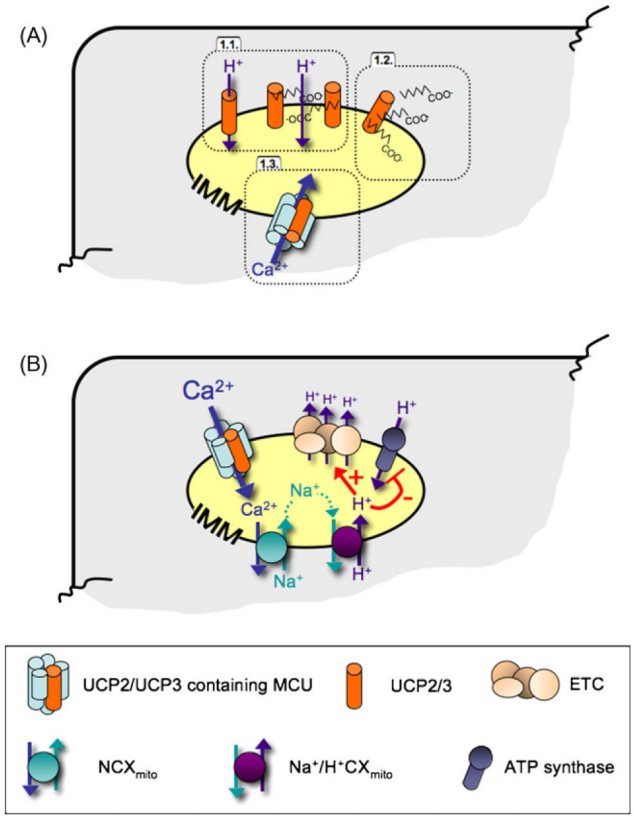

Figure 1.

Postulated molecular action of UCP2 and UCP3. (Panel A) The three proposed molecular actions of UCP2 and UCP3 are schematically illustrated as 1.1., establishing a H^+-leak that is most likely dependent on free fatty acids, 1.2., free fatty acid export from the mitochondria, and 1.3., contributing to mitochondrial Ca^{2+} uniport. (Panel B) While Ca^{2+} stimulates mitochondrial ATP production due to its stimulatory effect on various dehydrogenases [51—55], excessive mitochondrial Ca^{2+} sequestration causes matrix acidification and depolarization that is due to the mitochondrial Na^+/Ca^{2+} exchanger and the Na^+/H^+ exchanger that are activated subsequently to mitochondrial Ca^{2+} elevation. Accordingly, excessive mitochondrial Ca^{2+} uptake can cause uncoupling as a consequence of the mechanisms that lower matrix Ca^{2+} to avoid opening of the permeability transition pore, an initial step of mitochondriatriggered cell death.

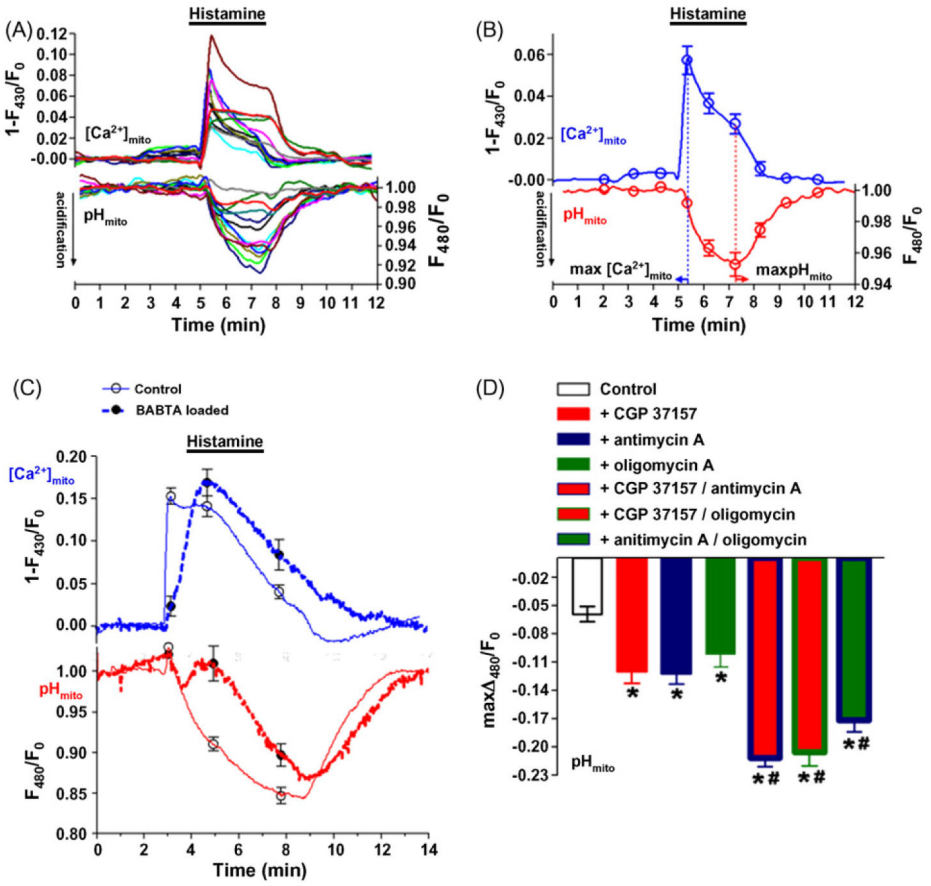

Figure 2.

Mitochondrial Ca^{2+} fluxes are accompanied by alterations in the pH of the mitochondrial matrix. The use of mitochondrial targeted ratiometric pericam allows simultaneous measurements of $[Ca^{2+}]_{mito}$ and changes of the pH of the mitochondrial matrix at the level of the single cell. EA.hy926 cells stably expressing RPmt were alternatively illuminated at 430 and 480 nm and emission was collected at 535 nm using a wide field imaging system [49,50]. As the signals at an excitation of 430 nm are largely insensitive to changing pH, but change promptly upon variations of the mitochondrial free Ca^{2+} concentration, these signals can be used to measure the time course of $[Ca^{2+}]_{mito}$-fluxes. In contrast, at an excitation of 480 nm the signal mainly follows changes of the mitochondrial matrix pH, whereas alterations of $[Ca^{2+}]_{mito}$ only marginally impact these values. (Panel A) Changes in $[Ca^{2+}]_{mito}$ of 14 different cells from 3 independent experiments upon stimulation with 100 μM histamine in the presence of 2 mM extracellular Ca^{2+} are presented (upper panel). The corresponding (same color) alterations of mitochondrial luminal pH are shown in the lower panel. (Panel B) Comparison of the kinetics of changes in $[Ca^{2+}]_{mito}$ (upper panel) with alterations in intramitochondrial pH (lower panel) indicate that following stimulation, mitochondrial Ca^{2+} elevation precedes mitochondrial acidification. (Panel C) The causative role of $[Ca^{2+}]_{mito}$ in triggering mitochondrial acidification becomes further evident by a comparison of the effect of histamine on mitochondrial Ca^{2+} and pH in control cells (continuous line; $n = 11$) with BAPTA/am loaded cells (dotted lines; $n = 12$). Ca^{2+} chelation slowed the kinetics of both parameters, while the sequence of appearance remained

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unchanged. (Panel D) The histamine-induced mitochondrial acidification (control; $n = 6$) was evaluated under different conditions using pharmacological tools. Inhibition of NCX_{mito} with 20 μM CGP 37157 strongly increased mitochondrial acidification (red column; $n = 8$). Similar findings were obtained using 10 μM antimycin A, a selective inhibitor of complex III (blue column, $n = 9$) or 5 μM oligomycin, an inhibitor of the ATP-synthase (green column, $n = 9$). These effects were amplified by combinations of the respecitive chemical compounds (CGP 37157/antimycin A, $n = 8$; CGP 37157/oligomycin, $n = 8$; antimycin A/oligomycin; $n = 9$).

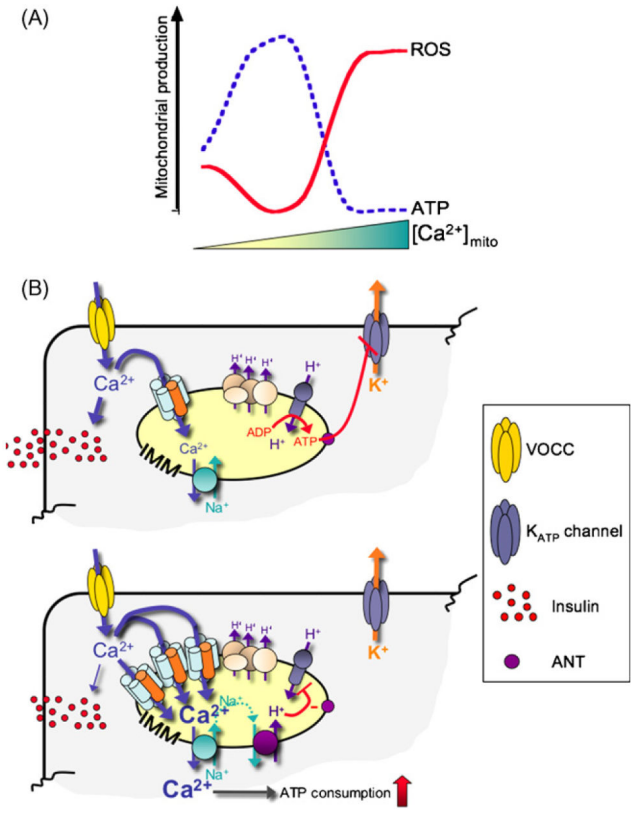

Figure 3.

Effect of mitochondrial Ca^{2+} on the energy balance of the cell and its subsequent effect on D-glucose-stimulated insulin secretion. (Panel A) We propose that increasing matrix $[Ca^{2+}]$ may cause a biphasic change in mitochondrial ATP and ROS production. At physiological Ca^{2+} elevations in the mitochondrial matrix, Ca^{2+} increases ATP formation and reduces ROS by activation of, e.g. MnSOD. However, at higher, presumably pathological Ca^{2+} elevations in the mitochondrial matrix, mitochondrial Ca^{2+} promotes ROS formation possibly via supramaximal stimulation of matrix dehydrogenases [162]. (Panel B, upper scheme) Ca^{2+} stimulates GSIS by two mitochondria dependent pathways. The first encompasses the stimulatory effect of matrix Ca^{2+} on ATP production that results in an elevated cytosolic ATP level, which, in turn, inhibits ATP-sensitive K^+ channels (K_{ATP} channels). This results in a depolarization of the cell membrane and consequently, activation of voltage gated Ca^{2+} channels (VOCC). In addition, entering Ca^{2+} directly stimulates insulin secretion. (Lower scheme) Upregulation of UCP2-dependent mitochondrial Ca^{2+} uniporter under conditions of high nutrition overflow, could increase the Ca^{2+} buffering capacity of subplasmalemmal mitochondria, buffering of Ca^{2+} influx and consequently, reducing Ca^{2+}-dependent insulin secretion. Moreover high $[Ca^{2+}]_c$ increases the cell's ATP consumption and causes mitochondrial uncoupling via excessive Ca^{2+} sequestration into the organelle, decreasing cytosolic ATP and so reducing insulin secretion.

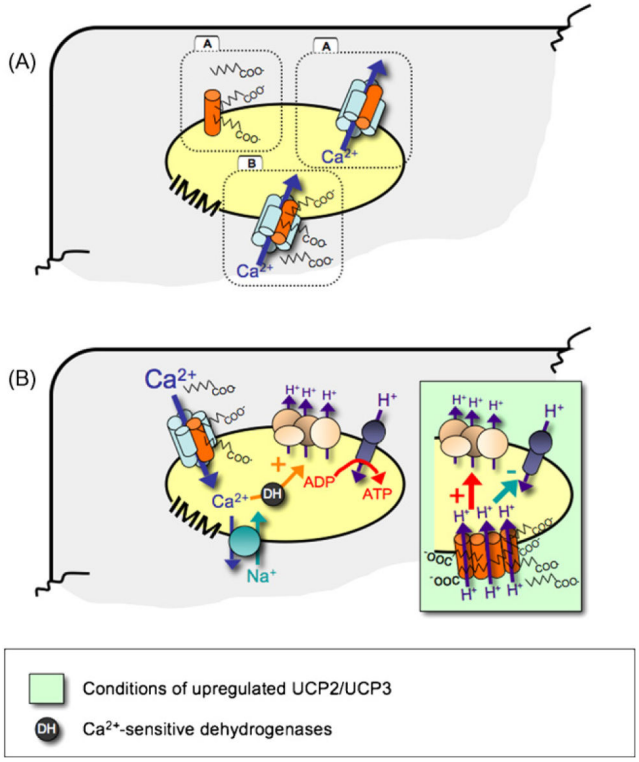

Figure 4.

Revised model of the molecular action of UCP2 and UCP3. (Panel A) A model is proposed in which UCP2 and UCP3 account for free fatty acid transport from the mitochondria and mitochondrial Ca^{2+} uniport. It remains to be investigated whether or not both molecular actions of UCP2/UCP3 are independent (A) or interrelated (B) functions of these proteins. (Panel B) Under physiological conditions we propose that UCP2 and UCP3 act as fatty acid carriers and as a fundamental part of the mitochondrial Ca^{2+} uniporter in so optimize and balance mitochondrial metabolic and signalling activity. However, upon nutrition overload, the upregulation of UCP2/UCP3 may disturb this homeostasis by, e.g. forming misfolded UCP proteins [22,23] and/or the lack of other, essential constituents of the mitochondrial Ca^{2+} uniporter [12], resulting in an accumulation of “free” UCP proteins in the membrane that may exhibit basal uncoupling activity, like many other members of the family of mitochondrial anion carrier protein family [15].

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Table 1

Overview on the reported evidences for an involvement of UCP2 and UCP3 in mitochondrial Ca^{2+} uniport [8]

Model*	Overexpression	siRNA	UCP2$^{-/-}$ mouse (liver)
Basal mitochondrial Ca^{2+}	Unchanged	Unchanged	n.d.
Mitochondrial Ca^{2+} uptake	Increased (even under de-polarized conditions)	Strongly reduced (rescue by UCP2 in UCP3 cells treated with siRNA against UCP3)	Absent (no ruthenium red-sensitive Ca^{2+} entry)
Capacity of the MCU	Increased (even under depolarized conditions)	Strongly reduced	Reduced
Ca^{2+} sensitivity of MCU	Unchanged	n.d.	n.d.
Basal $\Delta\psi_{mito}$	Unchanged	Unchanged	Unchanged
$\Delta\psi_{mito}$ upon stimulation/Ca^{2+} exposure	Unchanged	Unchanged	Unchanged
Basal pH_{mito}	Unchanged	Unchanged	n.d.
pH_{mito} upon stimulation	Decreased due to elevated $[Ca^{2+}]_{mito}$	Unchanged	n.d.
Basal ATP levels	Unchanged	n.d.	n.d.
ATP synthesis	Increased	n.d.	n.d.
Basal cytosolic Ca^{2+} concentration	Unchanged	Unchanged	n.d.
Intracellular Ca^{2+} mobilization	Unchanged	Unchanged	n.d.
Mitochondrial structure	Unchanged	Unchanged	n.d.
Focal contacts with the ER	Unchanged	Unchanged	n.d.
ER Ca^{2+} content	Unchanged	Unchanged	n.d.

*Most experiments were performed in human endothelial cells but were verified in HeLa, Hek293 and CHO cells.