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Review

Calcium and ATP handling in human NADH:Ubiquinone oxidoreductase deficiency

Federica Valsecchi [a,b], John J. Esseling [a], Werner J.H. Koopman [a,c,*], Peter H.G.M. Willems [a,c]

[a] Department of Membrane Biochemistry, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[b] Department of Pediatrics, Institute for Genetic and Metabolic Disease, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[c] Microscopical Imaging Centre, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

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ABSTRACT

Proper cell functioning requires precise coordination between mitochondrial ATP production and local energy demand. Ionic calcium (Ca^{2+}) plays a central role in this coupling because it activates mitochondrial oxidative phosphorylation (OXPHOS) during hormonal and electrical cell stimulation. To determine how mitochondrial dysfunction affects cytosolic and mitochondrial Ca^{2+}/ATP handling, we performed life-cell quantification of these parameters in fibroblast cell lines derived from healthy subjects and patients with isolated deficiency of the first OXPHOS complex (CI). In resting patient cells, CI deficiency was associated with a normal mitochondrial ($[ATP]_m$) and cytosolic ($[ATP]_c$) ATP concentration, a normal cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), but a reduced Ca^{2+} content of the endoplasmic reticulum (ER). Furthermore, cellular NAD(P)H levels were increased, mitochondrial membrane potential was slightly depolarized, reactive oxygen species (ROS) levels were elevated and mitochondrial shape was altered. Upon stimulation with bradykinin (Bk), the peak increases in $[Ca^{2+}]_c$, mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$), $[ATP]_c$ and $[ATP]_m$ were reduced in patient cells. In agreement with these results, ATP-dependent Ca^{2+} removal from the cytosol was slower. Here, we review the interconnection between cytosolic, endoplasmic reticular and mitochondrial Ca^{2+} and ATP handling, and summarize our findings in patient fibroblasts in an integrative model.

1. Introduction

Mitochondria generate ATP by the oxidative phosphorylation (OXPHOS) system located in the mitochondrial inner membrane (MIM; [62]). When ATP requirement increases, for instance when cell activity is triggered by a hormonal or electrical stimulus, OXPHOS-derived ATP production is increased to match cellular energy demand. A key player in this process is ionic calcium (Ca^{2+}), which enters the cytosol from the endoplasmic/sarcoplasmic reticulum (ER/SR) and/or the extracellular medium. As a consequence, the cytosolic [Ca^{2+}] ($[Ca^{2+}]_c$) is increased and Ca^{2+} is taken up by mitochondria to stimulate mitochondrial ATP production [56]. The ATP generated is exported to the cytosol to fuel cytosolic ATP-demanding processes like Ca^{2+} uptake in the ER by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and/or Ca^{2+} extrusion across the plasma membrane (PM) by PM Ca^{2+}-ATPases (PMCAs). As a consequence, $[Ca^{2+}]_c$ rapidly returns to basal levels again [23,63,88]. In this review, we discuss the interconnection between cytosolic, endoplasmic reticular and mitochondrial Ca^{2+} and ATP handling (section 2) and how mitochondrial CI deficiency may affect this coupling (section 3).

2. Interconnection between cellular Ca^{2+} and ATP handling

2.1. Hormone-induced Ca^{2+} release from the endoplasmic reticulum

The ER not only plays a key role in lipid and protein synthesis, folding and post-translational modification but also constitutes the main intracellular Ca^{2+} storage organelle [3,54]. Inside the lumen of the ER, part of the Ca^{2+} is free, whereas the remainder is bound to Ca^{2+}-binding proteins [24]. Estimation of the free luminal Ca^{2+} concentration ($[Ca^{2+}]_{ER}$) yielded values of 0.1–0.8 mM under resting conditions [63]. One of the main ER Ca^{2+}-binding proteins, calreticulin, is also involved in quality control and folding of newly-synthesized (glyco)proteins [72]. This suggests that these processes are under regulatory control of $[Ca^{2+}]_{ER}$ [6,10,26,48,66].

At any point in time, the total Ca^{2+} content of the ER (ERCa) depends on the amount of ER Ca^{2+} binding proteins and the balance

Abbreviations: $[ATP]_c$, cytosolic ATP concentration; $[ATP]_m$, mitochondrial ATP concentration; CI, complex I or NADH:ubiquinone oxidoreductase; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_{ER}$, ER Ca^{2+} concentration; $[Ca^{2+}]_m$, mitochondrial Ca^{2+} concentration; ERCa, endoplasmic reticulum Ca^{2+} content; ETC, electron transport chain; *F*, mitochondrial formfactor; HG, hyperglycemia; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; MCU, mitochondrial Ca^{2+} uniporter; *Nc*, number of mitochondria per cell; NDUF, NADH dehydrogenase ubiquinone flavoprotein; OXPHOS, oxidative phosphorylation; PM, plasma membrane; PMCA, plasma membrane Ca^{2+} ATPase; PMF, proton motive force; PTP, permeability transition pore; ROS, reactive oxygen species; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; VDAC, voltage-dependent anion channel

* Corresponding author. Department of Membrane Biochemistry (286), Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands. Tel.: +31 24 3614589; fax: +31 24 3616413.
E-mail address: w.koopman@ncmls.ru.nl (W.J.H. Koopman).

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between passive Ca^{2+} leak, Ca^{2+} release via specific Ca^{2+} channels such as the inositol 1,4,5 trisphosphate ($InsP_3$) receptor ($InsP_3R$) and the activity of the ER Ca^{2+}-pumps (SERCAs). Blocking of SERCA pumps in the absence of extracellular Ca^{2+}, induces an instantaneous increase in $[Ca^{2+}]_c$ in resting human skin fibroblasts [79], indicating that maintenance of ERCa requires continuous SERCA action to compensate for passive Ca^{2+} leak. It is likely that the ATP required to fuel the SERCAs is generated by mitochondria operating in close vicinity of the ER [54,56,63,88]. In this way, cells also maintain a low resting $[Ca^{2+}]_c$, which is not only a prerequisite for Ca^{2+} signaling, but also prevents possible toxic effects of Ca^{2+} like ATP precipitation, DNA breakdown and activation of Ca^{2+}-dependent proteases [47].

Many hormones trigger the production of $InsP_3$ via binding to a heterotrimeric G protein-activating receptor at the PM (Fig. 1B). The GTP-bound α-subunit of the G protein then stimulates the action of PM-bound phospholipase C beta (PLC-β), which cleaves phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) into 1,2-diacylglycerol (DAG), an activator of protein kinase C (PKC), and $InsP_3$. The latter molecule diffuses into the cytosol to stimulate the $InsP_3R$-mediated release of Ca^{2+} from the ER into the cytosol. As a result, $[Ca^{2+}]_c$ increases and, in healthy cells, is rapidly returned to pre-stimulus levels by mainly SERCA and/or PMCA action. In many cell types, hormone-induced cell activation is mediated by periodic rises in, often local, $[Ca^{2+}]_c$ to avoid the toxic side effects of Ca^{2+} (see above) and, more importantly, to allow high-fidelity signal transduction [18,25]. Thus, this oscillatory signaling mode, as it is generally referred to, enables coding of the signaling information in the frequency, amplitude and/or shape of the $[Ca^{2+}]_c$ rises and is, in general, only observed when low (physiological) concentrations of hormone are used [6,35,36,69]. The $InsP_3R$, exists as homo and/or heterotetramers [49]. In mammals, three $InsP_3R$ isoforms have been identified ($InsP_3R_1$, $InsP_3R_2$, $InsP_3R_3$) that are regulated by $[Ca^{2+}]_c$, $[Ca^{2+}]_{ER}$, protein kinases and specific binding proteins [21]. The latter include homer, protein 4.1N, huntingtin-associated protein-1A, protein phosphatases (PPI and PP2A), RACK1, ankyrin, chromogranin, carbonic anhydrase-related protein, IRBIT, Na^+/K^+-ATPase, and ERp44. This suggests that $InsP_3Rs$ form macro signal complexes that function as centers of signaling cascades [49].

2.2. The ER and mitochondria are in close vicinity

Evidence has been presented that at least part of the ER is in close vicinity of mitochondria (Fig. 1A). This juxtaposition creates an ER–mitochondrial 'synapse' that is thought to allow efficient exchange of Ca^{2+} and metabolites [1,11,13,20,22,45,54,57,60,67]. However, there is also evidence arguing against mitochondria-ER contacts being a general Ca^{2+} shunting mechanism [58]. For instance, artificial relocalization of mitochondria to the perinuclear region, which is rich in ER, does not affect mitochondrial Ca^{2+} uptake in response to stimulated Ca^{2+} release from internal stores. Additionally, mitochondria are highly mobile in cells, suggesting that: (i) ER–mitochondrial contacts are either formed between immobile parts of the ER and immobile mitochondria, (ii) mitochondria-connected parts of the ER move in parallel with mitochondria, or (iii) ER–mitochondrial contacts only exist transiently [58]. Furthermore, mitochondria can exist as small bean-shaped organelles and as more elongated tubular structures. Moreover, they can form intricate (sub)reticula [4,39,73]. Therefore, nature, functionality and amount of ER–mitochondrial contacts most probably depend on the type of cell and/or its metabolic condition. Taken together, it appears that a close proximity between ER and mitochondria alone is not sufficient for rapid Ca^{2+} transfer between the both organelles and that the latter requires assembly of an appropriate molecular machinery [58]. In this respect, recent evidence suggested that the ER–mitochondrial synapse is maintained by 10–25 nm tethers, physically connecting both organelles [13]. These tethers were shown to consist of the mitochondrial outer membrane (MOM)-protein VDAC1 (voltage-dependent anion channel 1 or porin), the chaperone glucose-related protein 75 (grp75 or mitochondrial heat shock protein 70; mtHSP70),

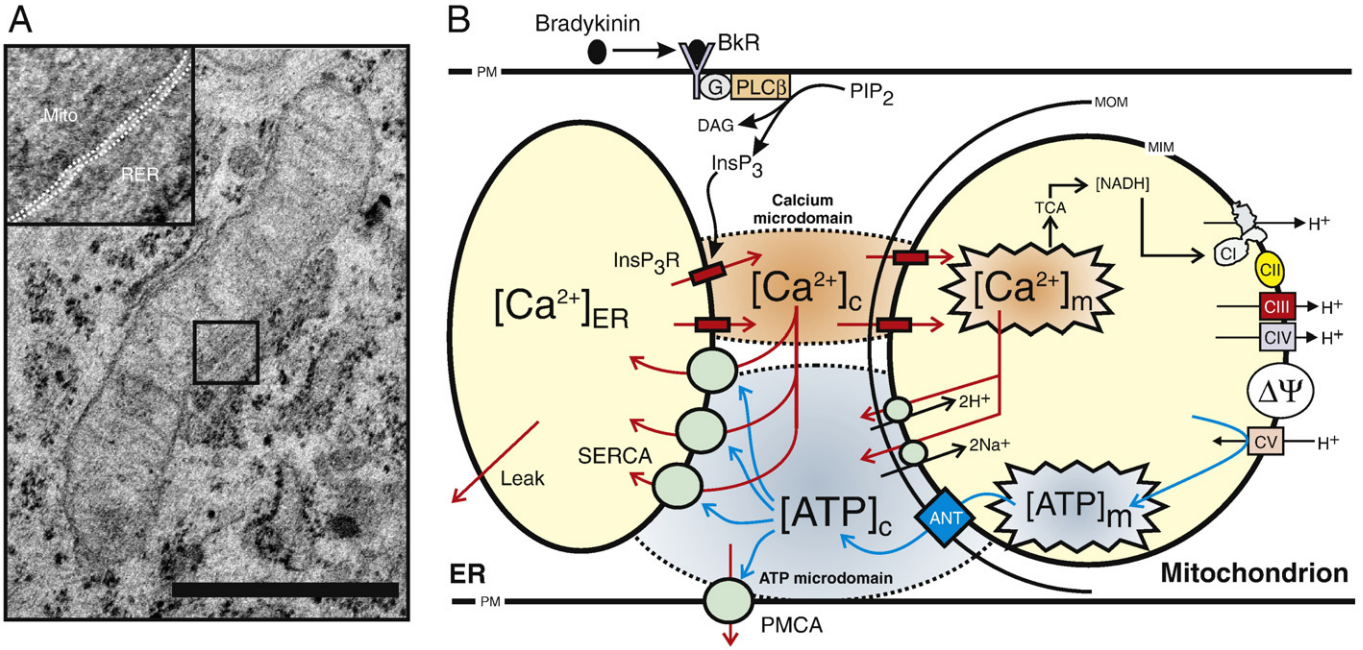

Fig. 1. Interconnection between ER, cytosolic and mitochondrial Ca^{2+} and ATP handling. (A) Contrast-optimized electron microscopy image of mitochondria in a typical healthy human skin fibroblast (CT-5120) showing the close proximity of a mitochondrion and parts of the rough ER (RER; inset). The scale bar indicates 1 μm (for details see: [40–42]. (B) Cartoon depicting the interconnection between Ca^{2+} and ATP homeostasis in the cytosol, ER and mitochondria during hormone-stimulation (see text for details). Abbreviations: ANT, adenine nucleotide translocase; $[ATP]_c$, cytosolic ATP concentration; $[ATP]_m$, mitochondrial ATP concentration; BkR, bradykinin-receptor; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_{ER}$, endoplasmatic reticulum Ca^{2+} concentration; $[Ca^{2+}]_m$, mitochondrial Ca^{2+} concentration; DAG, diacylglycerol; G, G-protein; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, inositol 1,4,5-trisphosphate receptor; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; PDHs, pyruvate dehydrogenases; PIP_2, phosphatidylinositol 4,5-bisphosphate; PM, plasmamembrane; PMCA, plasmamembrane Ca^{2+}-ATPase; PLCβ, phospholipase C-β; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase.

and the $InsP_3R$ [67]. In agreement with its putative role in ER–mitochondria tethering, overexpression of VDAC in HeLa cells and skeletal myotubes enhanced the amplitude of the agonist-induced rise in $[Ca^{2+}]_m$ and shortened the delay between the increase in $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ [55]. Functionally, VDAC-overexpressing cells were more susceptible to ceramide-induced cell death, suggesting that increased mitochondrial Ca^{2+} uptake stimulates the process of apoptosis. Similarly, overexpression of a grp75 mutant lacking the mitochondrial presequence also enhanced mitochondrial Ca^{2+} accumulation, implying that this protein plays a role in Ca^{2+} handling independent from its chaperone activity in the mitochondrial matrix [67]. Other recent evidence suggested that the ER protein sigma-1 receptor (Sig-1R), implicated in neuroprotection, carcinogenesis, and neuroplasticity and found to be located at the mitochondria-associated ER membrane (MAM), senses $[Ca^{2+}]_{ER}$ and might thus be involved in the regulation of ER–mitochondria Ca^{2+} signals [28]. Finally, very recent evidence implicated mitofusin 2, a mitochondrial dynamin-related protein demonstrated to be enriched at the ER–mitochondria interface, in the regulation of mitochondrial Ca^{2+} uptake. It was found that its down-regulation disturbed ER–mitochondria interactions leading to a decreased mitochondrial Ca^{2+} uptake in response to $InsP_3$ generating stimuli [46]. It appears that the distance between ER and mitochondrion, crucial for ER–mitochondrial Ca^{2+} transmission, is (co) regulated by $[Ca^{2+}]_c$ and determines the susceptibility to mitochondrial Ca^{2+} overload and opening of the mitochondrial permeability transition pore [13]. Free (local) $[Ca^{2+}]_c<100$ nM favors ER–mitochondria dissociation, whereas $[Ca^{2+}]_c>1$ μM favors close association between these organelles [85]. This suggests that ER subdomains are less closely associated with mitochondria in resting cells, whereas association occurs during cell stimulation.

2.3. Mitochondrial Ca^{2+} uptake, release and ATP generation

During cell stimulation, cytosolic Ca^{2+} rapidly enters the mitochondrial matrix by action of the $\Delta\psi$-dependent mitochondrial Ca^{2+} uniporter (MCU) leading to an increase in $[Ca^{2+}]_m$ [54,56]. The MCU is half-maximally activated at a $[Ca^{2+}]_c$ of ~10–20 μM and has a maximal Ca^{2+} flux of $\sim 2\cdot 10^4$ $Ca^{2+}\cdot s^{-1}$ per MCU molecule [45]. The close ER–mitochondrial juxtaposition allows the $[Ca^{2+}]_c$ within the Ca^{2+} microdomain (Fig. 1B) to become sufficiently high for MCU activation. Using confocal imaging of Rhod-2-loaded HeLa cells, Bootman et al. demonstrated that mitochondria will accumulate Ca^{2+} regardless whether it is released from the ER by $InsP_3$, enters across the PM or leaks from the ER [11]. However, in agreement with the $InsP_3R$ being involved in ER–mitochondrial tethering (see above), the rate of mitochondrial Ca^{2+} uptake was greatest for $InsP_3$-evoked Ca^{2+} signals.

Interestingly, when glomerular afferent arteriolar smooth muscle cells (PGASMC) were chronically pre-treated with Transforming Growth Factor-β (TGF-β), subsequent hormone-induced $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ rises were decreased [52]. Although TGF-β down-regulated both $InsP_3R_1$ and $InsP_3R_3$, ER Ca^{2+} storage, mitochondrial distribution and ER–mitochondrial contacts were not affected. It was concluded that TGF-β causes uncoupling of mitochondria from ER Ca^{2+} release by decreasing the $InsP_3R$-mediated Ca^{2+} efflux, thus hampering the build-up of the $[Ca^{2+}]_c$ within the ER–mitochondrial Ca^{2+} microdomain.

The decay phase of $[Ca^{2+}]_m$ is much slower than the MCU-mediated rising phase, and is primarily mediated by Na^+/Ca^{2+} (NCX) exchange across the MIM. The NCX is thought to be electrogenic because it exchanges 1 Ca^{2+} for 3 Na^+ with a K_m for Na^+ of 8 mM [45]. MCU stimulation by 4,4′,4″-(4-propyl-[^{1}H]-pyrazole-1,3,5-triyl)trisphenol (PPT) or kaempferol increased the histamine-induced ER Ca^{2+} release in HeLa cells and fibroblasts [76]. This effect was enhanced by simultaneous inhibition of NCX with the benzothiazepine CGP37157, suggesting that mitochondrial Ca^{2+} uptake and release control feedback inhibition of $InsP_3Rs$ within the ER–mitochondrial Ca^{2+} microdomain (Fig. 1B). Interestingly, PPT and kaempferol induced $[Ca^{2+}]_c$ oscillations in previously silent fibroblasts. This suggests that also in the absence of hormone stimulation, the occurrence of oscillatory Ca^{2+} signals depends on the rates of mitochondrial Ca^{2+} uptake and release, which modulate $InsP_3R$ activation. In HeLa cells, inhibition of mitochondrial NCX by CGP37157 during histamine stimulation changed the pattern of histamine-induced $[Ca^{2+}]_c$ oscillations [29]. Similarly, CGP37157 increased the frequency of $[Ca^{2+}]_c$ oscillations in human fibroblasts with spontaneous activity and induced the generation of oscillations in cells without spontaneous activity. These findings suggest that mitochondrial NCX directly modulates $InsP_3$-induced Ca^{2+} release and thereby controls $[Ca^{2+}]_c$ oscillations.

Within the mitochondrial matrix, an elevation in $[Ca^{2+}]_m$ stimulates respiration and increases mitochondrial [ATP] ($[ATP]_m$) [33,45,79,80,88]. Mitochondrial ATP production is carried out by the OXPHOS system, which consists of five MIM-embedded multi-protein complexes [62]. The system is primarily fueled by NADH and $FADH_2$, generated by the tricarboxylic acid (TCA) cycle. Electrons are abstracted from NADH and $FADH_2$ at complex I (CI or NADH: Ubiquinone oxidoreductase; EC 1.6.5.3) and II (CII or succinate dehydrogenase; EC 1.3.5.1), respectively, and donated to complex III (CIII or ubiquinol cytochrome *c* reductase; EC 1.10.2.2) via the MIM-bound electron transporter ubiquinone. Next, cytochrome *c* transports the electrons to complex IV (CIV or cytochrome *c* oxidase; EC 1.9.3.1), where they are donated to molecular oxygen (O_2) leading to the formation of water (H_2O). At CI, CIII and CIV protons are translocated across the MIM to create a proton-motive force (PMF) that consists of a chemical (ΔpH) and electrical component ($\Delta\psi$). This PMF is utilized at complex V (CV or ATP synthase; EC 3.6.3.14) to form ATP from ADP and P_i.

Three key dehydrogenases of the TCA cycle (pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase), as well as CV, are activated by Ca^{2+} [56,68]. Computational analysis also predicts that multi-site OXPHOS activation is required for efficient matching of mitochondrial ATP production with cellular demand [43]. The K_a for activation of the TCA cycle dehydrogenases is within the range of 0.7–1 μM [45], compatible with the observed increases in $[Ca^{2+}]_m$ [9,79].

Once produced, mitochondrial ATP is transported out of the mitochondrion by the adenine nucleotide translocase (ANT; [34]). This transporter exchanges ATP against ADP across the MIM and has also been proposed to be involved in mitochondrial Ca^{2+} homeostasis. The latter is illustrated by the fact that ANT overexpression reduced the histamine-induced peak increase in $[Ca^{2+}]_m$ in HeLa cells [87]. This effect was paralleled by alterations in mitochondrial shape and also reduced ERCa, resulting in a small decrease in peak $[Ca^{2+}]_c$ during histamine stimulation.

Within the cytosol, the ATP generated is used to fuel, among others, the SERCA and/or PMCA (Fig. 1B), which return $[Ca^{2+}]_c$ to the pre-stimulus level [54]. The $[Ca^{2+}]_c$ in close vicinity of the SERCA/PMCA also affects the action of these pumps. In this way, mitochondrial ATP production and Ca^{2+} uptake may exert local control on PMCA action and SERCA activity in the adjacent ER [63].

3. ER, cytosolic and mitochondrial Ca^{2+} and ATP handling in isolated complex I deficiency

3.1. Isolated complex I deficiency

CI is the largest OXPHOS complex (~1 MDa) and constitutes the entry point of electrons in the electron transport chain (ETC; [62]). Structurally, CI is L-shaped and consists of 45 different subunits, 14 of which are essential for catalytic function [8]. These 'core' subunits are encoded by genes on both the mitochondrial (mtDNA) and nuclear

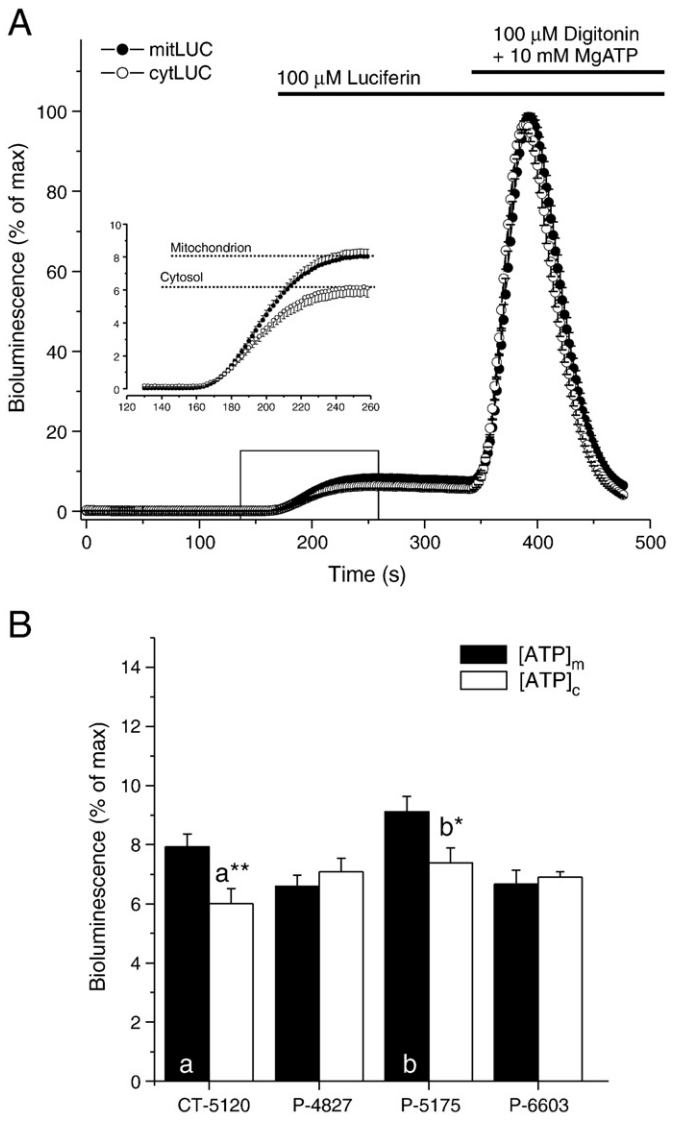

Fig. 2. Resting mitochondrial and cytosolic [ATP] in CI-deficient patient fibroblasts. (A) Calibration of average luciferase (LUC) bioluminescence signals in the mitochondrial matrix (mitLUC; closed symbols) and cytosol (cytLUC; open symbols) in a population (25,000 cells) of healthy human skin fibroblast (CT-5120). Following application of the LUC co-factor luciferin, a steady state level of bioluminesce was reached. This signal was expressed as percentage of the maximal bioluminesce signal obtained after permeabilization of the cells with digitonin in the presence of a saturating [ATP] (10 mM). This approach allowed comparison (see inset) of the resting [ATP] in the mitochondrion and cytosol. Bioluminescence signals were recorded with a photomultiplier system as described previously [79]. (B) Comparison of the resting [ATP] in the mitochondrial matrix (closed bars) and cytosol (open bars) in control cells (CT) and 3 different patient cell lines. Asterisks indicate significant differences with the indicated columns (*$p<0.05$; **$p<0.01$). More details are given in the text and Table 1.

DNA (nDNA). The seven mtDNA-encoded core subunits (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) all are part of the membrane arm of CI, whereas the nDNA-encoded ones (NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7 and NDUFS8) are mainly localized in the matrix-protruding arm of the complex. The remaining 31 subunits are all nDNA-encoded and their function is largely unknown. *In vivo* assembly of functional CI is thought to occur by stepwise combination of pre-assembled modules at the MIM [14] and requires assistance of assembly factors, six of which have currently been identified (NDUFAF1/CIA30, NDUFAF2/B17.2L/NDUFA12L/Mimetin, C6orf66, C8orf38, C20orf7 and Ecsit; [17,44,51,53,64,82,83]).

Deficiency of CI (OMIM 252010) was first described in humans 30 years ago [50]. A reduced enzyme activity of this complex, for diagnostic purposes usually determined in skeletal muscle and cultured skin fibroblasts, leads to multi-system disorders affecting predominantly organs and tissues with a high-energy demand like the brain, heart, and skeletal muscle [61]. Although patients with isolated CI deficiency due to nDNA mutations may present with a variety of signs and symptoms, Leigh disease is the most frequent, accounting for almost half of the cases [32,44]. Leigh disease is an early-onset (mostly during the first year of life) fatal neurodegenerative disorder that is typically characterized by symmetrical lesions of necrosis and capillary proliferation in variable regions of the central nervous system. Clinical signs and symptoms include optic atrophy, ophthalmoparesis, muscular hypotonia, ataxia and dystonia. In all cases, the disease is fatal (most children do not reach the age of 1 year).

Currently, mutations in 12 different nDNA-encoded CI subunits have been linked to isolated CI deficiency (NDUFS1, NDUFS2, NDUFS4, NDUFS6, NDUFS7, NDUFS8, NDUFV1, NDUFV2, NDUFA1, NDUFA2, NDUFA8, NDUFA11; [5,19,30,32,44]). Additionally, pathological mutations in five CI assembly factors (NDUFAF1/CIA30, NDUFAF2/B17.2L/NDUFA12L/Mimetin, C6orf66, C8orf38 and C20orf7) have been reported [2,17,44,51,53,59,64,83]. Currently, effective treatment strategies for isolated CI deficiency are lacking due to the limited insight into its cytopathology. Therefore, in the past years, we systematically investigated the possible consequences of nDNA-encoded CI mutations in patient fibroblasts. In doing so, we found aberrations in Ca^{2+} and ATP homeostasis, ROS handling and mitochondrial morphology (summarized in: [39,88]).

3.2. Mitochondrial Ca^{2+} and ATP handling in patient skin fibroblasts

Our previous studies with a cohort of skin fibroblast lines from patients with isolated CI deficiency revealed that CI mutations primarily lead to a reduced amount of fully assembled and catalytically active CI by decreasing the rate of assembly and/or disturbing the stability of the holo-complex [41,74,77].

Quantification of ERCa in resting cells, revealed that the ER contained less Ca^{2+} in patient cells, which displayed a reduced peak increase in $[Ca^{2+}]_c$, $[Ca^{2+}]_m$ and $[ATP]_m$ upon stimulation with the $InsP_3$-generating hormone bradykinin (Bk; see below and: [79,80,88]). This suggests that $[ATP]_c$ might be too low in resting patient cells to allow sufficient ATP fueling of the SERCAs. To investigate this possibility, we developed a protocol for quantification of $[ATP]_c$ and $[ATP]_m$, based on a previously described method for endothelial cells (supplement of [71]). Briefly, the bioluminescent ATP sensor luciferase (LUC) was expressed in the cytosol (cytLUC) or mitochondrial matrix (mitLUC; using the COX8 mitochondrial targeting sequence) of skin fibroblasts from a healthy subject and 3 patients harboring a mutation in one of the nDNA-encoded CI genes (*NDUFS4*, *NDUFS7*, *NDUSF8*; Table 1). MitLUC and cytLUC were introduced into the cells using the baculoviral system [12], made suitable for protein expression in human skin fibroblasts [79]. Luciferase catalyzes the luminescent reaction of luciferin with ATP and oxygen. Fig. 2A shows that upon addition of luciferin, the bioluminescence signal gradually increased to a steady level, reflecting the resting ATP concentration. Subsequent permeabilization by digitonin in the presence of a saturating concentration of ATP yielded the maximal signal which was used for normalization of the resting signal. As depicted in Fig. 2B, the resting values of $[ATP]_c$ and $[ATP]_m$ did not significantly differ between healthy and patient fibroblasts. Although the resting values of $[ATP]_m$ and $[ATP]_c$ were not altered in CI-deficient patient fibroblasts, it cannot be excluded that local decreases in $[ATP]_c$, in the ER–mitochondrial Ca^{2+} microdomain, occur and are responsible for the observed reduction in resting ERCa (Fig. 1B).

Concerning the Ca^{2+} buffering capacity in the ER lumen, Western blot analysis of whole-cell homogenates showed that the calreticulin protein amount was increased rather than decreased in typical patient

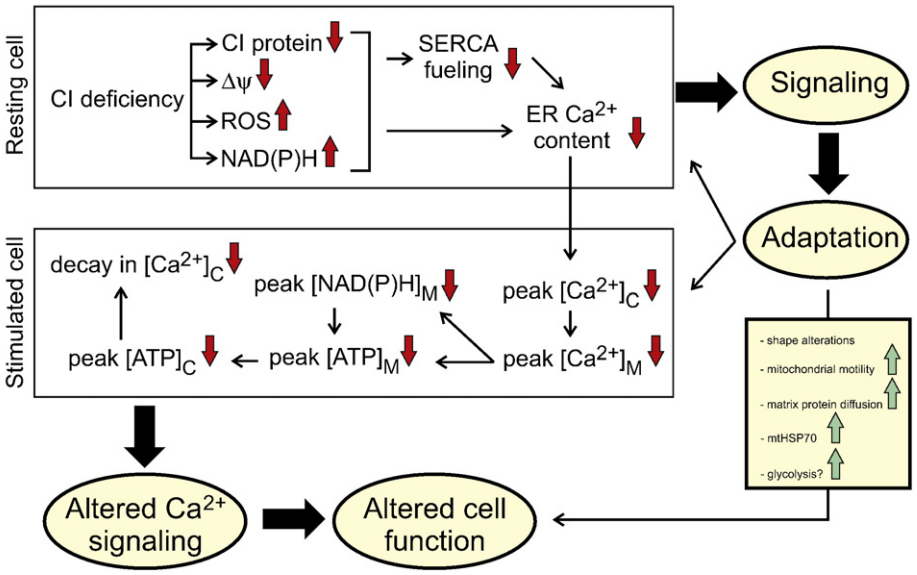

Fig. 3. Cell biological consequences of isolated CI deficiency in patient fibroblasts. Typical aberrations observed in fibroblasts of patients with isolated CI deficiency obtained by quantitative life-cell microscopy and bioluminometry (see text for details).

fibroblasts. Because calreticulin is the predominant Ca^{2+} binding protein of the ER, this finding disfavors the idea that the reduction in resting ERCa is due to a decrease in ER Ca^{2+} buffering capacity [88]. Measurement of ER Ca^{2+} uptake under saturating [ATP], revealed that the maximum SERCA-pump capacity was similar in control and patient fibroblasts. Additionally, the amount of SERCA2b protein, was

Table 1
Characteristics of typical complex I-deficient fibroblast cell lines.

Parameter	CT-5120	P-4827	P-5175	P-6603
Subunit	n.a.	NDUFS4	NDUFS7	NDUFS8
Subunit features	n.a.	Accessory	Core	Core
Mutation	n.a.	R106X	V122M	R94C
CI activity	113	**58**	**68**	**18**
PN	17	7*	15*	15*
Clinical phenotype	n.a.	L/LL	L/LL	L/LL
Resting Cell				
$\Delta\Psi$[a]	100 ± 0.2 (1033)	n.d.	**91 ± 1** (384)	**89 ± 1** (121)
$[NAD(P)H]_m$[a]	100 ± 2 (143)	n.d.	112 ± 7 (40)	**170 ± 6** (62)
ATP: $[ATP]_m$[b]	100 ± 5 (22)	83 ± 5 (6)	115 ± 7 (17)	84 ± 6 (3)
ATP: $[ATP]_c$[b]	100 ± 9 (20)	118 ± 8 (6)	123 ± 8 11)	115 ± 3 (3)
Calcium: $[Ca^{2+}]_c$[a]	100 ± 4 (32)	109 ± 5 (23)	109 ± 5 (21)	106 ± 6 (20)
Calcium: ERCa[a]	100 ± 1 (81)	n.d.	**73 ± 2** (16)	**85 ± 6** (17)
ROS: Et[a]	100 ± 1 (526)	**196 ± 6** (93)	**151 ± 5** (94)	**222 ± 7** (147)
ROS: CM-DCF[a]	100 ± 2 (412)	n.d.	**212 ± 15** (76)	**275 ± 20** (117)
ROS: Mitochondrial mitochondrial roGFP1[a]	38 ± 2 (42)	n.d.	37 ± 3 (20)	37 ± 3 (34)
ROS: Cytosolic cytosolic roGFP1[a]	15 ± 1 (23)	n.d.	16 ± 1 (22)	24 ± 2 (29)
ROS: lipid peroxidation[a]	100 ± 3 (99)	n.d.	87 ± 3 (99)	111 ± 3 (90)
Shape: Formfactor formfactor (*F*)[a]	100 ± 1 (1200)	96 ± 7	**117 ± 3** (214)	**120 ± 4** (69)
Shape: mitos per cell (*Nc*)[a]	100 ± 2 (1200)	**177 ± 25** (20)	**98 ± 10** (214)	**167 ± 9** (69)
Stimulated Cell				
Calcium: peak $[Ca^{2+}]_c$[a]	100 ± 3 (32)	**82 ± 4** (23)	**80 ± 4** (21)	**91 ± 4** (20)
Calcium: peak $[Ca^{2+}]_m$[b]	100 ± 2 (11)	n.d.	**76 ± 5** (3)	**89 ± 3** (5)
ATP: peak $[ATP]_m$[b]	100 ± 2 (31)	**33 ± 5** (3)	**57 ± 5** (3)	**74 ± 1** (6)
Calcium: Ca^{2+} removal[a]	100 ± 5 (32)	**169 ± 12** (23)	**164 ± 10** (21)	**134 ± 6** (20)

The data for healthy control (CT) and CI-deficient patient (P) cell lines, except $[ATP]_m$ and $[ATP]_c$ in resting cells, was taken from previous studies (summarized in: [39] and [88]). The numbers indicate the designation of the cell line within the Nijmegen Centre for Mitochondrial Disorders.
CI activity is expressed as percentage of the lowest value in a collection of control cell lines [62]; PN: passage number, an asterisk indicates the passage number after arrival at the Nijmegen Centre for Mitochondrial Disorders.
Clinical phenotype: HCEM, hypertrophic cardiomyopathy and encephalomyopathy; L, Leigh syndrome; LL, Leigh-like syndrome.
Resting Cell: $\Delta\Psi$, mitochondrial membrane potential measured with tetramethyl rhodamine methyl ester (TMRM; [15,42]); $NAD(P)H_m$, mitochondrial NAD(P)H autofluorescence [78]; ATP, see Fig. 2 and main text; calcium: pre-stimulatory fura-2 ratio and ER calcium content (ERCa; [80,81]); ROS: Et, rate of ethidium formation [78], CM-DCF, rate of chloromethyl dichlorofluorescein formation [38,78]; roGFP1, oxidation status (% oxidized) of cytosolic and mitochondria-targeted redox-sensitive GFP [78]; Lipid peroxidation, detected with C11-BODIPY$^{581/591}$ [78]; Shape: formfactor is a combined measure of mitochondrial length and degree of branching [42].
Stimulated Cell: peak values of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ measured with fura-2 and mitochondria-targeted aequorin, respectively [80,81]; Peak ATP measured using mitochondria-targeted luciferase [80,81]. The rate of cytosolic Ca^{2+} removal is determined using the fura-2 signal and given by the time constant of a mono-exponential fit (larger means slower).
Statistics: the data are expressed in percentage of the average control value ± standard error. Values in bold are significantly different from control value ($p<0.05$).
Abbreviations: CI, complex I; n.a., not appropriate; n.d., not determined; NDUFS, NADH dehydrogenase ubiquinone flavoprotein; *Nc*, number of mitochondria per cell.
[a] The number between brackets indicates the numbers of cells analyzed on at least 2 different days.
[b] The number between brackets indicates the number of independent assays on at least 2 different days.

not altered in typical patient cells [88], further supporting the conclusion that ERCa is decreased because of reduced SERCA fueling by mitochondrial ATP.

Typically, when patient cells were stimulated with Bk, transient increases in $[Ca^{2+}]_c$, $[Ca^{2+}]_m$ and $[ATP]_m$ were observed, which peaked at lower values than in healthy control cells [79,80,88]. Regression analysis of the patient data, revealed that the peak values of $[Ca^{2+}]_c$, $[Ca^{2+}]_m$ and $[ATP]_m$ were linearly correlated, suggesting that the magnitude of the $[Ca^{2+}]_c$ transient is the primary determinant of the $[Ca^{2+}]_m$-induced increase in $[ATP]_M$. This is compatible with a mechanism in which $[Ca^{2+}]_m$ stimulates mitochondrial ATP production [33]. Given the fact that mitochondrial Ca^{2+} uptake is $\Delta\psi$-dependent [56,75,79], the linear relationship between peak $[Ca^{2+}]_c$ and peak $[Ca^{2+}]_m$ furthermore suggests that the small $\Delta\psi$ depolarization observed in patient fibroblasts does not significantly contribute to the observed alterations in Bk-stimulated mitochondrial Ca^{2+} and ATP handling. Quantification of the decay rate of the $[Ca^{2+}]_c$ transient revealed a halftime ($t_{1/2}$) that was inversely proportional to the peak $[ATP]_m$, suggesting that mitochondria-generated ATP serves to fuel SERCA-mediated Ca^{2+} reuptake by the ER [80,81,88].

Recent studies using isolated cardiomyocytes of mice with tissue-specific knockout of mitochondrial transcription factor A (Tfam), revealed that action potential-mediated $[Ca^{2+}]_c$ transients, measured with the fluorescent indicator Fluo-3, were smaller and faster than control [89]. Moreover, both the Ca^{2+} content of the sarcoplasmic reticulum and the expression level of the Ca^{2+}-binding protein calsequestrin-2 were found to be reduced in Tfam knockout hearts. These results indicate that although the consequences of Tfam ablation for the stimulus-induced $[Ca^{2+}]_c$ increase in mice cardiomyocytes resemble those in CI-deficient human skin fibroblasts, the underlying mechanism is different.

3.3. Cell biological consequences of complex I deficiency: an integrative model

In addition to Ca^{2+} and ATP handling, we also have investigated the consequences of isolated CI deficiency on CI assembly, OXPHOS protein expression, mitochondrial membrane potential ($\Delta\psi$), reactive oxygen species (ROS) levels, thiol redox status, lipid peroxidation, NAD(P)H level, mitochondrial morphology, motility and intra-matrix protein diffusion (Fig. 3; [37,40,41,77,79,88]).

In patient cells (see Table 1 for typical examples), reduced expression of the CI holo-enzyme was paralleled by a (slightly) depolarized $\Delta\psi$, and increased ROS and NAD(P)H levels. Because the reduction in CI levels was inversely proportional to cellular ROS levels, it appears that decreasing numbers of active complexes generate increasing amounts of ROS not because of the presence of a mutated subunit but as a consequence of a decrease in cellular CI activity. The alternative explanation that increasing numbers of partially assembled complexes are responsible for the observed increase in ROS production is not supported by our finding that chronic rotenone treatment increased rather than decreased the amount of fully assembled CI [77].

In a recent study, we investigated the effect of chronic treatment with Trolox, a water-soluble derivative of vitamin E, on cellular ROS levels and expression and activity of fully assembled CI [41]. It was found that ROS levels were dramatically reduced and CI expression and activity were variably increased in healthy and patient fibroblasts. This suggests that the amount of active CI is under regulatory control of the cell's oxidative balance. By ratioing the Trolox-induced increase in CI activity and CI amount, we determined whether newly generated CI holo-complexes were catalytically active. The results obtained revealed that apart from the amount also the intrinsic activity of the complex can be significantly decreased in nDNA-inherited isolated CI deficiency. The finding that Trolox treatment increased the amount of CI might provide an experimental basis for the use of antioxidants to mitigate the deficiency. However, it is to be expected that such a treatment is only beneficial to patients with a predominant expression rather than intrinsic catalytic defect of the complex.

As discussed above (section 3.2), the CI deficiency-induced mitochondrial dysfunction leads to a reduced ERCa in resting cells, most probably by reduced ATP fueling of SERCAs. Alternatively, ERCa in patient cells might be reduced by functional impairment of the SERCAs and/or $InsP_3$ receptor by increased ROS levels [7,31,65,86]. However, maximal SERCA-pump capacity in patient fibroblasts was normal (section 3.2) and ERCa displayed a strong linear correlation with the Bk-induced $InsP_3R$-mediated peak increase in $[Ca^{2+}]_c$, disfavoring a role for $InsP_3R$ malfunction [80]. Given the established signaling role of Ca^{2+} in the ER lumen, the reduced ERCa might affect intra-ER and/or cytosolic signal transduction pathways. In this respect, it has been proposed that intra-ER Ca^{2+} not only functionally regulates Ca^{2+}-binding chaperones responsible for intra-ER protein folding, but also the expression of these chaperones once released from the ER by cell stimulation [27]. Similarly, ERCa might regulate proteins involved in ER stress responses like the UPR (unfolded protein response) and ERAD (ER-associated degradation). To determine if such mechanisms are operational in CI-deficient patient fibroblasts, further investigations are needed.

It is likely that the elevated ROS/NADH(P)H triggers activation of adaptive responses [16,70]. This might be related to the observed changes in mitochondrial shape, motility, intra-matrix protein diffusion and mtHSP70 expression in patient cells [4,39,40]. We previously argued that these alterations reflect a switch to a (more) glycolytic mode of ATP generation in patient fibroblasts [40]. Similarly, in 143B osteosarcoma cybrid cells harboring pathogenic mtDNA point mutations in $tRNA^{Leu}$, the major OXPHOS defect was efficiently compensated by increased anaerobic glycolysis, so that the total ATP production rate was preserved [84]. The latter is compatible with the normal resting $[ATP]_m$ and $[ATP]_c$ observed in our patient cells (section 3.2). The 143B cybrid cells displayed an increased ROS production, which was not paralleled by the induction of antioxidant defense systems or substantial oxidative damage. Comparison of a cohort of 10 healthy and 10 CI-deficient patient cell lines also revealed no detectable differences in thiol redox status, glutathione/glutathione disulfide content, or extent of lipid peroxidation [78]. This indicates that fibroblasts of patients with isolated CI deficiency maintain their thiol redox status despite the marked increase in ROS production. Similar to our patient cells, $\Delta\psi$ was depolarized in 143B cybrid cells and this was associated with a disturbed mitochondrial Ca^{2+} homeostasis [84].

In summary, the data presented in this review highlight that mutations in nDNA-encoded CI subunits have 4 major consequences: (i) they reduce the amount and activity of fully assembled and active CI, (ii) they increase ROS levels, (iii) they cause aberrations in Ca^{2+}/ATP handling and, (iv) they trigger an adaptive response. Currently, strategies targeting these consequences in order to mitigate CI deficiency are under way in our laboratory.

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