The antioxidant Trolox restores mitochondrial membrane potential and Ca\(^{2+}\)-stimulated ATP production in human complex I deficiency

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Abstract Malfunction of mitochondrial complex I caused by nuclear gene mutations causes early-onset neurodegenerative diseases. Previous work using cultured fibroblasts of complex-I-deficient patients revealed elevated levels of reactive oxygen species (ROS) and reductions in both total Ca\(^{2+}\) content of the endoplasmic reticulum (ER\(_{\text{Ca}}\)) and bradykinin (Bk)-induced increases in cytosolic and mitochondrial free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{C}}\); [Ca\(^{2+}\)]\(_{\text{M}}\)) and ATP ([ATP]\(_{\text{C}}\); [ATP]\(_{\text{M}}\)) concentration. Here, we determined the mitochondrial membrane potential (\(\Delta\psi\)) in patient skin fibroblasts and show significant correlations with cellular ROS levels and ER\(_{\text{Ca}}\), i.e., the less negative \(\Delta\psi\), the higher these levels and the lower ER\(_{\text{Ca}}\). Treatment with 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) normalized \(\Delta\psi\) and Bk-induced increases in [Ca\(^{2+}\)]\(_{\text{M}}\) and [ATP]\(_{\text{M}}\). These effects were accompanied by an increase in ER\(_{\text{Ca}}\) and Bk-induced increase in [Ca\(^{2+}\)]\(_{\text{C}}\). Together, these results provide evidence for an integral role of increased ROS levels in complex I deficiency and point to the potential therapeutic value of antioxidant treatment.

Keywords Reactive oxygen species · Oxidative stress · Mitochondrial membrane potential · Endoplasmic reticulum Ca\(^{2+}\) content · Mitochondrial disease · Antioxidant · Human skin fibroblasts

Abbreviations

\(\Delta\psi\) mitochondrial membrane potential
Bk bradykinin
CI complex I or NADH:ubiquinone oxidoreductase
OXPHOS oxidative phosphorylation
ROS reactive oxygen species
TMRM tetramethyl rhodamine methyl ester
ER endoplasmic reticulum
ER\(_{\text{Ca}}\) ER total Ca\(^{2+}\) content
[Ca\(^{2+}\)]\(_{\text{C}}\) and [Ca\(^{2+}\)]\(_{\text{M}}\) cytosolic and mitochondrial free Ca\(^{2+}\) concentration
[ATP]\(_{\text{M}}\) mitochondrial ATP concentration

Introduction

Mitochondria are double membrane-bound organelles that do not only constitute the ‘cellular power plants’ but also are crucially involved in survival, apoptosis, redox control, Ca\(^{2+}\) homeostasis, and many metabolic and biosynthetic pathways [1]. To fulfill these diverse functions, mitochon-
ria require a highly membrane potential across their inner membrane ($\Delta \psi$), which is maintained by the action of the three proton pumps (complexes I, III, and IV) of the electron transport chain. Together with $F_0/F_1$-ATP-synthase (complex V), which uses the resulting electrochemical proton gradient to generate ATP, and complex II of the electron transport chain, which oxidizes FADH$_2$, these proton pumps constitute part of the oxidative phosphorylation (OXPHOS) system, which accounts for the majority of cellular ATP production.

Complex I (EC 1.6.5.3) is the largest OXPHOS complex and clinical presentations associated with its deficiency (MIM 252010) are heterogeneous ranging from severe multi-system diseases with neonatal death (e.g., Leigh Disease) to isolated myopathies [2]. Complex I consists of seven subunits encoded by the mitochondrial DNA and 38 by that of the nucleus [3]. As to the nuclear genome, disease-causing mutations have been identified in the genes of core subunits NDUFS1, NDUFS2, NDUFS3, NDUFS7, and NDUFS8 [4-9], supernumerary subunits NDUFS4, NDUFS6, NDUFAM1, and NDUFAM2 [10-13], and complex I assembly factors NDUFA2, NDUFAF2, and C6orf66 [14-16].

Detailed knowledge of the cytopathological consequences of complex I deficiency is prerequisite to understanding the clinical presentations and their heterogeneity, and developing therapeutic strategies [17]. To gain this knowledge, we study skin fibroblasts from patients with mutations in nuclear complex I genes. We demonstrated that these mutations can be associated with a reduction in Ca²⁺ content of the endoplasmic reticulum (ER$_{Ca}$) [18, 19]). Because OXPHOS depends on the electrochemical proton gradient, the latter might be altered in patient cells, leading to a reduced supply of ATP to ER Ca²⁺-pumps, thus explaining the observed reduction in resting ER$_{Ca}$. In agreement with this idea, we showed before that chronic inhibition of complex I by rotenone reduced $\Delta \psi$ and decreased ER$_{Ca}$ in healthy fibroblasts [20]. We furthermore showed that a reduction in ER$_{Ca}$ was paralleled by decreased amplitudes of the bradykinin (Bk)-induced increases in [Ca²⁺]$_{c}$, [ATP]$_{c}$, and [ATP]$_{M}$ [19]. Finally, we demonstrated that these aberrations were accompanied by a decrease in the rate of cytosolic Ca²⁺ removal during the decay phase of the Bk-induced [Ca²⁺]$_{c}$ transient [19]. Because Bk stimulates the release of Ca²⁺ from the ER into the cytosol from which Ca²⁺ is taken up by mitochondria to stimulate the OXPHOS process, these data suggest that the reduction in resting ER$_{Ca}$ is responsible for the decrease in Bk-stimulated mitochondrial ATP production, which, in turn, results in a lower cytosolic Ca²⁺ removal rate. Evidence comes from experiments with, on the one hand, an inhibitor of mitochondrial Na⁺-Ca²⁺ exchange, which was shown to normalize both the Bk-induced increase in [ATP]$_{M}$ and the rate of cytosolic Ca²⁺ removal in patient fibroblasts [18], and on the other hand a mitochondrial Ca²⁺ buffer, which decreased both parameters in fibroblasts of a healthy individual [21].

Apart from alterations in intracellular Ca²⁺ handling we observed elevated levels of reactive oxygen species (ROS) in fibroblasts of patients with a nuclear complex I gene defect [22, 23]. Because elevated ROS levels have been associated with a less negative $\Delta \psi$ [24, 25], and because a less negative $\Delta \psi$ might decrease mitochondrial ATP-dependent Ca²⁺ uptake by the ER (see above), we here investigated the relationship between $\Delta \psi$, elevated ROS levels and ER$_{Ca}$ in these patient fibroblasts.

**Materials and methods**

**Cell lines**

Fibroblasts were obtained following informed parental consent and according to the relevant Institutional Review Boards from skin biopsies of four healthy subjects and ten patients in whom an isolated complex I deficiency was confirmed in both muscle tissue and cultured fibroblasts (Table 1). Activity measurements were performed as described previously [26]. The activity of the complex was normalized against that of cytochrome c oxidase, which was in the control range, and expressed as percentage of the lowest control (0.11 nM/uM cytochrome c oxidase; [26]). All patients were negative with respect to mitochondrial DNA alterations previously associated with complex I deficiency. Fibroblasts were cultured in HEPES (25 mM)-buffered M199 medium (Invitrogen) supplemented with 10% fetal calf serum, 5 mg/l Tween 20, 100 IU/ml penicillin and 100 IU/ml streptomycin.

**Fluorescence imaging of $\Delta \psi$**

$\Delta \psi$ was measured by digital imaging microscopy of fibroblasts loaded with tetramethyl rhodamine methyl ester (TMRM) as described in detail before [27]. During measurements, cells were maintained in a HEPES–Tris medium (pH 7.4) containing 132 mM NaCl, 4.2 mM KCl, 5.5 mM D-glucose, 1 mM MgCl$_2$, 1 mM CaCl$_2$ and 10 mM HEPES.

**Luminescence monitoring of [Ca²⁺]$_{M}$ and [ATP]$_{M}$**

[Ca²⁺]$_{M}$ and [ATP]$_{M}$ were determined by luminometry of fibroblasts transduced with a baculovirus encoding mitochondrial targeted forms of wild-type aequorin (mtAEQ) and luciferase (mtLUC) as described previously [18, 19, 21]. During [ATP]$_{M}$ measurements, cells were superfused
Fluorescence imaging of \([\text{Ca}^{2+}]_C\)

\([\text{Ca}^{2+}]_C\) was measured by digital imaging microscopy of fibroblasts loaded with fura-2 as previously described [18, 19, 21]. Briefly, cells were challenged with 2',5'-d(i tert-buty l)-1,4-benzohydroquinone (BHQ; 10 μM), a specific inhibitor of the ER \(\text{Ca}^{2+}\) ATPase, in the absence of external \(\text{Ca}^{2+}\) (no \(\text{Ca}^{2+}\) added to the extracellular medium and in the presence of 0.5 mM EGTA). Under these conditions, \(\text{Ca}^{2+}\) that passively leaks out of the ER is not resequestered, resulting in a rapid increase in \([\text{Ca}^{2+}]_C\). The peak increase in fluorescence emission ratio after treatment with BHQ was used as a measure of \(\text{ER}_{\text{Ca}}\).

**Chemicals and statistics**

Culture material and TMRM were from Invitrogen (Breda, The Netherlands). Trolox was from Fluka (Buchs, Switzerland). All other reagents were from Sigma (Zwijndrecht, the Netherlands). Values from multiple experiments were expressed as average±S.E.M and statistical significances were assessed by Student's \(t\)-test.

**Results**

\(\Delta \psi\) is reduced in complex-I-deficient patient fibroblasts Using our recently developed method for microscopic quantification of \(\Delta \psi\) in cultured human skin fibroblasts [13, 27, 28].

**Statistics:** values significantly different from control C-5120 (\(p<0.05\)) are depicted in italics

\(N\) indicates the number of cells analyzed, \(N.d.\) not determined, \(C\) indicates control cells

\(\text{a}\) Numerals indicate the designation of the cell lines within the Nijmegen Center for Mitochondrial Disorders (NCMD)

\(\text{b}\) Determined relative to complex IV and expressed as percentage of lowest control [35]

\(\text{c}\) Expressed as % of the value for control C-5120

\(\text{d}\) \(\text{ER}_{\text{Ca}}\), expressed as % of the value for control C-5120 [23, 25]

\(\text{e}\) Expressed as % of the value for control C-5120 [27, 28]

with HEPES/Tris medium containing 5 μM beetle luciferin (Promega, Madison, WI). Prior to \([\text{Ca}^{2+}]_M\) measurements, mtAEQ was reconstituted with 5 μM native coelenterazine (Promega) in serum-free M199 medium for 1 h at 37°C. The wild-type aequorin-native coelenterazine pair measures \(\text{Ca}^{2+}\) concentrations in the 0.1-10 μM range and mtAEQ photon emission was converted off-line to \(\text{Ca}^{2+}\) concentration by using of a computer algorithm based on the \(\text{Ca}^{2+}\) response curve of wild-type aequorin [28]. Bk was added through the superfusion medium.

Determination of \(\text{ER}_{\text{Ca}}\)

\(\text{ER}_{\text{Ca}}\) was determined by digital imaging microscopy of fibroblasts loaded with fura-2 as described before [18, 19, 21].
we here show that $\Delta \psi$ is variably decreased in fibroblast lines of ten complex-I-deficient patients compared to four healthy individuals (Fig. 1a). For each cell line, the average TMRM intensity per mitochondrial pixel is expressed relative to that of C-5120 healthy fibroblasts measured on the same day. Linear regression analysis revealed the absence of any correlation between $\Delta \psi$ and residual complex I activity.

$\Delta \psi$ is related to cellular ROS levels and ERCa. We showed previously that cellular ROS levels are elevated to a variable extent in our whole cohort of complex-I-deficient patients (Table 1; [22, 23]). Linear regression analysis revealed a negative correlation between ROS levels and $\Delta \psi$ (Fig. 1b). We have also shown that, similar to cellular ROS levels, ERCa is variably decreased in these patient fibroblasts (Table 1; [19]). Again a significant, this time positive correlation was observed (Fig. 1c).

**Chronic Trolox treatment normalizes $\Delta \psi$** Based on the significant correlation between ROS levels and $\Delta \psi$, as shown in Fig. 1b, we concluded that lowering of oxidant levels may restore the electrochemical proton gradient across the inner mitochondrial membrane. We previously demonstrated that chronic treatment of patient fibroblasts with Trolox, a water-soluble analogue of vitamin E, reduced ROS levels and increased the amount of fully assembled complex I [30]. Figure 2 shows that chronic Trolox (300 µM, 72 h) fully restored $\Delta \psi$ in S4-5260 and S7-5175 patient cells, which were among those displaying the largest decrease in $\Delta \psi$ (Fig. 1a). Trolox did not affect $\Delta \psi$ in healthy fibroblasts.

**Chronic Trolox treatment dose-dependently increases ERCa** A reduced $\Delta \psi$ may have severe consequences for mitochondrial energy metabolism and thus for cellular processes, such as ER Ca$^{2+}$ uptake, that depend hereupon.
This prompted us to investigate the possibility that Trolox may restore ER_{Ca} in our patient fibroblasts. Indeed, chronic Trolox (72 h) dose-dependently increased ER_{Ca} to ∼135% of the value obtained with untreated C-5120 healthy cells in both these and S4-5260 patient fibroblasts (Fig. 3). The half-maximal effect was reached at ∼85 µM Trolox. In sharp contrast, acute application of Trolox (500 µM; 30 min) neither changed ER_{Ca} in healthy (n=22 cells) nor increased it in patient (n=21 cells) fibroblasts (data not shown). The S4-5260 patient cell line was selected because it was among those with the largest decrease in ∆ψ and ER_{Ca}.

Chronic Trolox treatment restores aberrant Ca^{2+} and ATP handling during bradykinin stimulation. In a final series of experiments, we assessed the effect of chronic Trolox on the Bk-induced increase in [Ca^{2+}]_c, the ensuing increases in [Ca^{2+}]_M and [ATP]_M and the rate of cytosolic Ca^{2+} removal. Figure 4a shows that chronic Trolox dose-dependently increased the amplitude of the Bk (1 µM)-induced increase in [Ca^{2+}]_C to ∼125% of that in untreated C-5120 healthy cells in both these and S4-5260 patient cells. The half-time of Ca^{2+} removal (t_{1/2}) decreased from 22.5±0.8 s (n=28 cells) and 14.6±0.6 s (n=28 cells) for untreated and Trolox-treated patient fibroblasts, respectively. This means that Trolox also normalized the slower cytosolic Ca^{2+} removal rate in patient cells. Trolox did not alter t_{1/2} in healthy fibroblasts (t_{1/2}=14.2±0.8 s and 14.5±0.6 s for 24 untreated and 31 Trolox-treated healthy cells, respectively).

Chronic Trolox did not affect the Bk-induced peak increase in [Ca^{2+}]_M in healthy fibroblasts (Fig. 4b). In S4-5260 patient cells, however, chronic Trolox dose-dependently increased this parameter to the same value as in untreated healthy cells. Chronic Trolox was also without effect on the Bk-induced peak [ATP]_M increase in healthy cells, whereas it normalized this parameter in patient cells (Fig. 4c).

Discussion

NADH:ubiquinone oxidoreductase or complex I forms a major entry point of electrons into the mitochondrial OXPHOS system and its malfunction is associated with a wide variety of clinical presentations [2]. To understand the cellular mechanisms underlying these syndromes, we study cultured skin fibroblasts of a unique cohort of patients with inherited complex I deficiency of nuclear origin [20, 31, 32]. One of our most striking findings is that the amount of fully assembled complex I is markedly decreased in patient fibroblasts, indicating that in addition to a likely decrease in intrinsic catalytic activity also its assembly and/or stability is compromised [22, 30, 32]. Other important findings include increased ROS levels and disturbances of Ca^{2+} and...
ATP homeostasis both at rest and during stimulation [20, 31]. It is presently unknown how these changes are exactly brought about in these patient fibroblasts. Here, we show that ∆ψ is significantly reduced in our cohort of patient fibroblast lines. At first glance, the most likely explanation would be a decrease in proton translocation. However, the OXPHOS system is most probably not working at its maximum capacity in resting fibroblasts and may, therefore, very well be able to maintain ∆ψ even when the maximum capacity of its first complex is decreased due to a lower expression and/or decreased intrinsic catalytic activity. Given the strong correlation between ROS levels and ∆ψ, i.e., the higher these levels, the less negative ∆ψ, the above finding could also be explained in that increased ROS levels induce a proton leak [33]. The relevance of the consequent ∆ψ decrease would be to try and prevent excessive ROS production. We also show that ∆ψ and ERCa are correlated, i.e., the less negative ∆ψ, the lower ERCa. Because ∆ψ is an important determinant for the rate of mitochondrial ATP production, this result is in agreement with our idea that fueling of ER Ca²⁺-pumps with mitochondrial ATP is reduced in resting patient fibroblasts. Importantly, our previous results argue against the idea that the reduction in ERCa is due to increased ROS-induced Ca²⁺ leak [18].

The other major finding is that chronic Trolox normalizes ∆ψ in patient fibroblasts without affecting this parameter in healthy cells. This result is consistent with the above-described mechanism in which elevated ROS levels cause an increase in mitochondrial proton leak. Additional support comes from work demonstrating that Trolox is an efficient inhibitor of such a proton leak [34]. Normalization of ∆ψ likely restores cellular processes that are disturbed by its disease-associated reduction. Among these processes, adequate delivery of mitochondrial ATP to ER Ca²⁺-pumps is of crucial relevance. Strikingly, chronic Trolox increased ERCa in both healthy and patient fibroblasts. Although the increase in healthy cells was unexpected in view of the lack of effect of chronic Trolox on ∆ψ, it should be noted that the increase in ERCa observed with 100 µM Trolox was much more pronounced in patient cells, suggesting a dual mechanism involving normalization of ∆ψ (only in patient cells) and modification of a hitherto unknown process (both in healthy and patient cells). Such a dual mechanism is consistent with the idea that elevated ROS levels hamper proper fueling of ER Ca²⁺-pumps primarily by their effect on ∆ψ. Future research will reveal whether the ∆ψ-independent mechanism involves a decrease in passive Ca²⁺ leak, an increase in Ca²⁺ buffering capacity in the lumen of the ER and/or an increase in glycolytic ATP-dependent Ca²⁺ uptake.

The present result that the effect of chronic Trolox on ERCa was paralleled by a similar effect on the Bk-induced increase in [Ca²⁺]c is consistent with the linear relationship between both parameters in our cohort of patient fibroblast lines [19]. Similar to our finding, Trolox enhanced the bombesin-induced increase in [Ca²⁺]c in skin fibroblasts of patients with genetic and non-genetic forms of Alzheimer's disease [35]. Although chronic Trolox markedly enhanced the Bk-induced increase in [Ca²⁺]c in both healthy and patient fibroblasts, it did not affect the consequent increase in [Ca²⁺]M in healthy cells, whereas it dose-dependently normalized this parameter in patient cells. Calibration of the mtAEQ signal revealed that Bk increased [Ca²⁺]M to ~4.5 µM [18, 19, 21], whereas the maximum Ca²⁺ concentration that can be measured is 10 µM [28]. Therefore, the results obtained suggest that the capacity of mitochondria to take up Ca²⁺...
is restricted to a maximum that is reached with 1 µM Bk and cannot be exceeded even when the Bk-induced increase in \([Ca^{2+}]_C\) is further increased by the action of Trolox. Additional evidence for restricted \(Ca^{2+}\) uptake capacity was obtained in experiments with an inhibitor of mitochondrial \(Na^+-Ca^{2+}\) exchange. As expected, this inhibitor, CGP-37157, did not alter the Bk-induced increase in \([Ca^{2+}]_C\), whereas it normalized the Bk-induced increase in \([Ca^{2+}]_M\) in patient cells [18, 21]. Strikingly, CGP-37157 was without effect in healthy control cells, which is in agreement with the idea that 1 µM Bk maximally increases \([Ca^{2+}]_M\) in healthy fibroblasts.

In agreement with the widely accepted notion that hormone-induced increases in \([Ca^{2+}]_M\) stimulate mitochondrial ATP production [20], we show that chronic Trolox can normalize this process in patient fibroblasts without affecting it in healthy fibroblasts. Chronic Trolox normalized the rate of cytosolic \(Ca^{2+}\) removal during the decay phase of the Bk-induced \([Ca^{2+}]_C\) transient in patient fibroblasts without affecting this parameter in healthy cells. The same observation was reached using CGP-37157 [18, 21], whereas the opposite was observed with the mitochondrial \(Ca^{2+}\) buffer Rhod-2 [21]. These results, together with the fact that the Bk-induced increase in \([ATP]_M\) is tightly correlated with the rate of cytosolic \(Ca^{2+}\) removal for the whole cohort of patient fibroblasts [19], clearly link these two processes.

In conclusion, the present results support the idea that fueling with mitochondrial ATP of cytosolic processes that are stimulated by hormone-induced increases in \([Ca^{2+}]_M\), such as exocytotic release of neurotransmitters in nerve cells and contraction and relaxation of muscle cells, may be hampered in complex I deficiency. It is these two cell types that are among the most vulnerable in this disease [2]. However, the most important conclusion of this study is that the aberrations in \(Ca^{2+}\)-stimulated mitochondrial ATP production and the consequences thereof, such as observed in complex I deficiency, can be overcome with drugs that normalize the stimulus-induced increase in \([Ca^{2+}]_M\) by either blocking mitochondrial \(Ca^{2+}\) export (CGP-37157) or increasing mitochondrial \(Ca^{2+}\) uptake (Trolox). It remains an important future task to translate this cellular information to clinical practice. Because patients with inborn errors of mitochondrial metabolism present with variable disease onset and a broad range of clinical symptoms, disease-adapted or even individualized treatment strategies might be required. In this context, Trolox and CGP-37157 constitute interesting new candidates to be evaluated as treatment options.

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