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Mitigation of NADH: Ubiquinone oxidoreductase deficiency by chronic Trolox treatment

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ABSTRACT

Deficiency of mitochondrial NADH:ubiquinone oxidoreductase (complex I), is associated with a variety of clinical phenotypes such as Leigh syndrome, encephalomyopathy and cardiomyopathy. Circumstantial evidence suggests that increased reactive oxygen species (ROS) levels contribute to the pathogenesis of these disorders. Here we assessed the effect of the water-soluble vitamin E derivative Trolox on ROS levels, and the amount and activity of complex I in fibroblasts of six children with isolated complex I deficiency caused by a mutation in the *NDUFS1*, *NDUFS2*, *NDUFS7*, *NDUFS8* or *NDUFV1* gene. Patient cells displayed increased ROS levels and a variable decrease in complex I activity and amount. For control cells, the ratio between activity and amount was 1 whereas for the patients this ratio was below 1, indicating a defect in intrinsic catalytic activity of complex I in the latter cells. Trolox treatment dramatically reduced ROS levels in both control and patient cells, which was paralleled by a substantial increase in the amount of complex I. Although the ratio between the increase in activity and amount of complex I was exactly proportional in control cells it varied between 0.1 and 0.8 for the patients. Our findings suggest that the expression of complex I is regulated by ROS. Furthermore, they provide evidence that both the amount and intrinsic activity of complex I are decreased in inherited complex I deficiency. The finding that Trolox treatment increased the amount of complex I might aid the future development of antioxidant treatment strategies for patients. However, such treatment may only be beneficial to patients with a relatively small reduction in intrinsic catalytic defect of the complex.

1. Introduction

The oxidative phosphorylation (OXPHOS) system consists of five multi-protein complexes (I–V) and is essential for mitochondrial function [1–3]. OXPHOS deficiency is associated with a wide range of metabolic disorders, age-related diseases and certain forms of cancer [4–6]. OXPHOS deficiency can occur at any given age, but its onset is usually within the first 2 years of life. In 40% of the early-onset cases, the decrease in OXPHOS activity is associated with an isolated (25%) or combined (15%) deficiency of complex I [2]. Human complex I (NADH: ubiquinone oxidoreductase; EC 1.6.5.3) consists of 7 mitochondrial DNA-encoded (mtDNA) and 38 nuclear DNA-encoded (nDNA) subunits [7]. In the majority of early-onset patients, complex I deficiency (OMIM 252010) is caused by mutations in nDNA-encoded subunits [8–11]. Additionally, disease causing mutations have been found in nDNA-encoded complex I assembly factors like B17.2 L [12]. Recent work has shown that disease causing mutations may reduce the assembly/stability of holo complex I, resulting in a lower rate of NADH oxidation [13,14]. However, such mutations may also primarily decrease the intrinsic catalytic activity of the holocomplex [15].

At present, the pathophysiological mechanisms linking defects in complex I genes to cellular dysfunction and disease are poorly understood. Mutations in nDNA-encoded complex I subunits can alter mitochondrial shape and induce aberrations in mitochondrial and cellular Ca^{2+}/ATP handling [16–18]. Furthermore, evidence has been provided that the production of reactive oxygen species (ROS) is increased in isolated complex I deficiency [14,19–24]. If ROS are not adequately neutralized by the cell's antioxidant mechanisms, oxidative modifications of specific proteins may occur [25]. The latter may ultimately contribute to OXPHOS dysfunction. In-vitro assays with the flavoprotein fraction of bovine complex I revealed that NADH-induced superoxide production can cause specific oxidative modifications of the 51-kDa (NDUFV1) subunit, resulting in a decrease in electron

Abbreviations: BNP, blue native polyacrylamide gel electrophoresis; IGA, in-gel activity; NDUF, NADH dehydrogenase ubiquinone flavoprotein; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; WB, Western blot

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transfer activity [26]. In another study, using isolated perfused rat hearts, increases in complex I-mediated ROS production were found to be accompanied by thiol modifications of matrix-facing complex I subunits following ischemia reperfusion [21]. Finally, recent work revealed that increases in mitochondrial ROS production can lead to accelerated degradation of newly synthesized mitochondrial proteins [27]. It was suggested that consequent down-regulation of the most sensitive OXPHOS complexes may further increase mitochondrial superoxide production, which is compatible with the progressive nature of complex I deficiency.

In accordance with the above findings, antioxidants were found to improve OXPHOS function in superoxide dismutase 2 null mice [28]. Furthermore, chronic antioxidant treatment was shown to increase CV activity and ATP synthesis in cybrids containing the mtDNA of patients with the T8993G mtDNA mutation associated with impaired oxidative phosphorylation in NARP (neuropathy, ataxia and retinitis pigmentosa) and MILS (maternally inherited Leigh's syndrome) [29]. Likewise, chronic oral administration of vitamin E prevented the loss of mitochondrial function and reduced ROS-induced damage in aging mice [30]. These beneficial effects were paralleled by an increased lifespan, better neurological performance and higher exploratory activity. Regarding human complex I deficiency, patients have been found to respond differentially to antioxidant treatment (e.g. Ref. [31]).

Together, these findings indicate that superoxide and derived ROS may play an important role in the pathogenesis of disorders associated with defects of the OXPHOS system. Here, we assessed the relationship between cellular ROS levels and complex I expression and activity in nDNA-inherited isolated complex I deficiency. The data presented demonstrate that cellular ROS levels are significantly increased in patient cells and that this increase is associated with variable decreases in the amount and intrinsic catalytic activity of complex I. Most importantly, our finding that chronic treatment with a vitamin E derivative (Trolox) increases the amount of complex I might provide an experimental basis for the use of antioxidants to treat complex I deficient patients.

2. Materials and methods

2.1. Patient skin fibroblasts

Fibroblasts were obtained from skin biopsies of three healthy subjects and six complex I deficient children in the age range of 0–5 years following informed parental consent and according to the relevant Institutional Review Boards [32]. The deficiency was confirmed in both muscle tissue and cultured skin fibroblasts. Patients were screened for the presence of DNA alterations in each of the known nuclear-encoded complex I genes and found to carry mutations in either the *NDUFS1* (#6173), *NDUFS2* (#5170), *NDUFS7* (#5175), *NDUFS8* (#6603) or *NDUFV1* (#5866, #5171) gene (Table 1). All patients were shown to be negative with respect to mitochondrial DNA alterations associated with complex I deficiency. Fibroblasts were cultured in medium 199 with Earle's salt supplemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin and 100 IU/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cell cycle analysis revealed no differences between the various cell lines [16].

2.2. Quantification of cellular reactive oxygen species levels

Cellular reactive oxygen species levels were quantified as described previously [22,24]. Briefly, fibroblasts were incubated in HEPES–Tris medium (132 mM NaCl, 4.2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM D-glucose and 10 mM HEPES, pH 7.4) containing 1 μM 5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Molecular Probes) for 10 min at 37 °C. During this period, the acetate groups are cleaved by intracellular esterases yielding dichlorodihydrofluorescein (CM-H_2DCF), which is then oxidized by oxidants to form highly fluorescent dichlorofluorescein (CM-DCF). After loading, the cells were thoroughly washed and transferred to the stage of an Oz confocal microscope (Noran Instruments, Middleton, WI, USA). Routinely, CM-DCF fluorescence was monitored during 200 s at a 10 s-interval. After background correction, the rate of CM-DCF fluorescence increase during the first 150 s was determined as a measure of the cellular ROS level. On each measuring day, the average rate obtained with #5120 control fibroblasts was set at 100%, to which all values were related. All recordings were carried out at minimal laser intensity using identical hardware settings.

2.3. Blue native PAGE, Western blot analysis and in-gel activity measurement

Cultured skin fibroblasts were harvested by trypsinization, washed with ice-cold phosphate-buffered saline (PBS; Braun, Melsungen, Germany) and resuspended (approx. 2×10^6 cells) in 100 μl ice-cold PBS. For preparation of a mitochondria-enriched fraction, cells were incubated with 2 mg/ml digitonin (Biosciences Inc., La Jolla, CA, USA) in a final volume of 200 μl for 10 min on ice. Next, 1 ml ice-cold PBS was added followed by centrifugation (5 min; 10,000 ×*g*; 4 °C). Mitochondrial pellets were washed twice with 1 ml ice-cold PBS and stored overnight (−20 °C). Pellets were solubilized in 100 μl ACBT buffer (Fluka, Steinheim, Germany) containing 1.5 M aminocaproic acid and 75 mM Bis–Tris/HCl (pH 7.0). To extract mitochondrial protein complexes, 20 μl 10% (w/v) β-lauryl maltoside was added and the solution was incubated for 10 min on ice. After centrifugation (30 min; 10,000 ×*g*; 4 °C), 10 μl of BN sample buffer (Biorad Laboratories, Hercules, CA, USA) was added to the supernatant. Blue native PAGE, Western blotting using a monoclonal antibody against the NDUFA9 (39-kDa) subunit of complex I (Molecular Probes) at a dilution of 1:1000, and in-gel activity measurement were performed as described previously [33]. For quantitative analysis, gels were loaded with exactly 10 μg of mitochondrial protein.

Table 1
Control and patient fibroblasts

Cell line[a]	Mutated subunit	Mutation[b]	Clinical phenotype[c]	Reference
CT1 (#5120)	None	None	n.a.	[17]
CT2 (#5119)	None	None	n.a.	[16]
CT3 (#5118)	None	None	n.a.	[16]
P1 (#6603)	*NDUFS8*	R94C	L/LL	[16]
P2 (#6173)	*NDUFS1*	R557X/D618N	L/LD	[17]
P3 (#5170)	*NDUFS2*	R228Q	HCEM	[46]
P4 (#5866)	*NDUFV1*	R59X/T423M	MLM/LL	[47]
P5 (#5175)	*NDUFS7*	V122M	L/LL	[48]
P6 (#5171)	*NDUFV1*	R59X/T423M	MLM/LL	[47]

[a] CT and P indicate control and patient cell lines, respectively. Numbers indicate the designation of the cell lines within the Nijmegen Centre for Mitochondrial Disorders (NCMD).
[b] Mutations are given at the protein level.
[c] Clinical phenotype: L/LD, Leigh syndrome and leukodystrophy; L/LL, Leigh syndrome and Leigh-like syndrome; MLM, macrocephaly, leukodystrophy and myoclonic epilepsy; HCEM, hypertrophic cardiomyopathy and encephalomyopathy. Abbreviations: n.a., not appropriate.

2.4. Data analysis

After Western blotting, luminescent signals were quantitatively analyzed by exposing illumination films to the blots for different periods of time (5–180 s). Films that displayed sub-maximal, non-saturated, signals were scanned using a G690 Imaging Densitometer (Biorad). From these scans, the integrated optical density of each band was determined and background corrected. The resulting numerical values were normalized to those obtained with control cells on the same blot. For quantitative analysis of in-gel activity, gels were scanned directly. Numerical results were visualized using Origin Pro 7.5 (OriginLab Corp., Northampton, MA, USA) and presented as the mean ± SEM. Statistical differences were determined using either a two-population or one-population Student's *t*-test (Bonferroni corrected).

2.5. Chemicals

Culture materials were obtained from Invitrogen (Breda, The Netherlands), all other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

3. Results

3.1. Cellular ROS levels are decreased by the water-soluble vitamin E derivative Trolox

To quantify cellular ROS levels, fibroblasts of a healthy control subject (#5120) were loaded with non-fluorescent CM-H_2DCFDA (5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; 1 μM) for 10 min, thoroughly washed to remove excess CM-H_2DCFDA, and monitored for the conversion of intracellularly trapped CM-H_2DCF into fluorescent CM-DCF. Video-imaging microscopy revealed that CM-DCF fluorescence was uniformly distributed throughout the cell (Fig. 1A) and increased linearly with time (Fig. 1B; open symbols). Importantly, this linearity indicates that the amount of CM-H_2DCF is not rate-limiting [22,24]. Acute application of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) instantaneously decreased the rate of CM-DCF formation by ~50% (Fig. 1B; filled symbols and inset). We showed previously that acute application of exogenous hydrogen peroxide (H_2O_2) readily increased the rate of CM-DCF formation in human skin