Application of an Image Cytometry Protocol for Cellular and Mitochondrial Phenotyping on Fibroblasts from Patients with Inherited Disorders

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Abstract Cellular phenotyping of human dermal fibroblasts (HDFs) from patients with inherited diseases provides invaluable information for diagnosis, disease aetiology, prognosis and assessing of treatment options. Here we present a cell phenotyping protocol using image cytometry that combines measurements of crucial cellular and mitochondrial parameters: (1) cell number and viability, (2) thiol redox status (TRS), (3) mitochondrial membrane potential (MMP) and (4) mitochondrial superoxide levels (MSLs). With our protocol, cell viability, TRS and MMP can be measured in one small cell sample and MSL on a parallel one. We analysed HDFs from healthy individuals after treatment with various concentrations of hydrogen peroxide (H_2O_2) for different intervals, to mimic the physiological effects of oxidative stress. Our results show that cell number, viability, TRS and MMP decreased, while MSL increased both in a time- and concentration-dependent manner. To assess the use

of our protocol for analysis of HDFs from patients with inherited diseases, we analysed HDFs from two patients with very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency (VLCADD), one with a severe clinical phenotype and one with a mild one. HDFs from both patients displayed increased MSL without H_2O_2 treatment. Treatment with H_2O_2 revealed significant differences in MMP and MSL between HDFs from the mild and the severe patient. Our results establish the capacity of our protocol for fast analysis of cellular and mitochondrial parameters by image cytometry in HDFs from patients with inherited metabolic diseases.

Introduction

Cell culture techniques allow the study of cellular mechanisms in a controlled environment. They are relevant in a wide range of studies: molecular disease mechanisms, cell metabolism and gene expression, as well as for disease prognosis and monitoring the response of treatments (Lipman et al. 1992; Sandell and Sakai 2011; Makpol et al. 2012). Human dermal fibroblasts (HDFs) are primary cells that mimic the conditions *in vivo* and are obtained from a non-invasive skin biopsy (Rittié and Fisher 2005; Smith 2006; Sandell and Sakai 2011). HDFs provide invaluable information for characterization of mutation defects in patients with inherited diseases (Smith 2006; Jensen 2010; Burbulla and Krüger 2012; Fernández-Guerra et al. 2014). HDFs possess the genetic composition of patients, and although HDF cultures lack the heterotypic cell–cell interactions and the three-dimensional geometry of tissues, many pathways are expressed in culture environment (Palmfeldt et al. 2009; Freshney 2011; Brand and Nicholls 2011).

Studies of molecular disease mechanisms often reveal the involvement of mitochondria; mitochondria are critical

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for the viability and physiology of eukaryotic cells, especially for energy metabolism, cell cycle and programmed cell death (Burhans and Heintz 2008; Norberg et al. 2010; Brand and Nicholls 2011). Mitochondria also play a key role as regulators of cellular stress responses because they are the main producers of reactive oxygen species (ROS). At low concentrations, ROS regulate cellular signalling; however, at high concentrations, they cause damage to biomolecules (Valko et al. 2006; Murphy 2009). Thus, biomarkers for mitochondrial function are very informative parameters of cell physiology and prospective cellular viability.

There exists a variety of fluorescence-based techniques for cellular and mitochondrial phenotyping in intact cells, where mitochondria are present in their physiological environment (Brand and Nicholls 2011; Cottet-Rousselle et al. 2011). These techniques can detect cell subpopulations and study subcellular compartments in a cellular sample. One of these techniques is image cytometry (ICM) that has developed strongly in recent years due to advancements in CCD (charge-coupled device) cameras and LED (light-emitting diode) technology. ICM acquires images from cell samples producing quantitative fluorescence data from a high number of cells simultaneously, as well as gather morphological information like cell size and shape. The data analysis of the images allows for unambiguous identification of cells and cell aggregates based on their morphology or fluorescence labelling (Ozaki et al. 2010; Pierzchalski et al. 2010; Chan et al. 2011).

The purpose of the present study was to develop a cell phenotyping protocol that combines a set of cellular and mitochondrial parameters in a small cell sample, leading to a reduction in biological material, time and analytical variability. The analysed parameters were (1) cell number and viability, (2) thiol redox status (TRS), (3) mitochondrial membrane potential (MMP) and (4) mitochondrial superoxide levels (MSLs). This combination of phenotypic parameters can give a nuanced picture of mutation effects on cellular physiology in HDFs from patients with inherited diseases. To test the protocol, HDFs from healthy individuals were analysed with and without treatment with various concentrations of hydrogen peroxide (H_2O_2) at different time points. H_2O_2 is a stable ROS capable of triggering cellular stress responses when exogenously administered, thereby mimicking the physiological effects of oxidative stress (Choi et al. 2009).

To evaluate the capacity of this protocol for analysing effects of genetic changes, we analysed HDFs from patients with very long-chain acyl-CoA dehydrogenase (VLCAD, EC 1.3.99.13) deficiency (VLCADD) (MIM#201475). VLCADD is characterized by a genetic defect in the *ACADVL* gene (MIM#609575) leading to a defective activity

of the mitochondrial enzyme VLCAD that is involved in fatty acid β -oxidation. This mitochondrial disorder results in a variety of clinical phenotypes ranging from relatively mild to life threatening (Houten and Wanders 2010; Schiff et al. 2013). Previous studies have suggested a relation between VLCADD and oxidative stress (Tucci et al. 2010; Cardoso et al. 2013). Therefore, we used HDFs from VLCADD patients to determine whether our protocol could detect this mitochondrial and oxidative stress implication.

Materials and Methods

Reagents

4',6'-Diamidino-2-phenylindole (DAPI), VitaBright-48 (VB-48), 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (JC-1), acridine orange (AO), propidium iodide (PI), Hoechst 33342 (Hoechst) and cell lysis buffer were purchased from ChemoMetec (Allerød, Denmark). Antimycin, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), dimethylsulfoxide (DMSO) and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich (Denmark). Hank's balanced salt solution (HBSS) with calcium and magnesium and MitoSOXTM Red mitochondrial superoxide indicator (MitoSOX) were purchased from Invitrogen (Denmark).

Patients and Healthy Individuals

HDFs isolated from two healthy individuals were Cambrex #CC-2509 and ATCC #CRL-2450 which annotated NHDF-01 and NHDF-02, respectively. Patients were referred for genetic diagnosis of *ACADVL* gene (GenBank reference sequence NM_000018.3) on the basis of elevated blood C14:0, C14:1, C16:0 and C18:0 acylcarnitines. Patient one (P1) (c.[689C>T;428_467del]) presented a mild phenotype and harbours a missense mutation (p.Thr230Ile) together with a deletion leading to premature stop codon (p.Gly143Alafs*61). Patient two (P2) (c.[685C>T;685C>T]) presented a severe phenotype and harbours a nucleotide change that generates a premature stop codon (p.Arg229*). The nomenclature of the mutations is according to the Human Genome Variation Society (HGVS) (<http://www.hgvs.org/mutnomen/>) and has been revised with the software Mutalyzer (<https://mutalyzer.nl/>). The samples have been deidentified according to the regulations of the Danish Ethical Committee.

Cell Culture and H_2O_2 Treatment

HDFs were used below passage 15 and cultured with Dulbecco's modified Eagle's medium (DMEM) (Lonza,

Denmark) supplemented with 10% (v/v) foetal bovine serum (Biological Industries, Denmark), 0.29 mg/mL L-glutamine (Leo Pharmaceutical, Denmark) and 1% penicillin/streptomycin (Leo Pharmaceutical, Denmark), at 37°C in 5% CO₂. *Mycoplasma sp.* test was performed routinely (PromoKine, Heidelberg, Germany).

HDFs were seeded at 80% confluence in a T25 flask (Nunc, Roskilde, Denmark) 24 h prior to treatment with 0, 2 or 4 mmol/L H₂O₂ in serum-free DMEM for different time intervals. Then, HDFs were trypsinized and aliquoted to measure cell number and viability, TRS and MMP. MSL was measured in a parallel experiment in a 6-well plate (Nunc, Roskilde, Denmark). At least three independent experiments were performed for each measurement.

Image Cytometry

Measurements of cellular fluorescence were performed with an NC-3000 image cytometer with a 2-chamber NC-Slide A2™ (ChemoMetec, Allerød, Denmark) and a minimum of 5,000 cells analysed. The NC-3000 software (ChemoMetec, Allerød, Denmark) was used for fluorescent image acquisition, image analysis, subpopulation definition and quantification and data visualization.

Cell Number and Viability Detection Using DAPI as Nuclear Staining

HDFs (2×10^5 cells) were mixed with cell lysis buffer (1:1) and DAPI (25 µg/mL) for determination of the total cell concentration (Ct). Another aliquot was mixed with DAPI (25 µg/mL) for the non-viable cells concentration (Cnv). DAPI fluorescence was detected using peak excitation at 365 nm and emission at 470/55. Cell viability was calculated according to the following formula, viability = (Ct – Cnv)/Ct, and expressed as percentage.

Thiol Redox Status (TRS) Measurement Using VitaBright-48

VB-48 is a live permeable dye that reacts with intracellular thiol groups forming a fluorescent compound (Skindersoe et al. 2012). An aliquot of HDFs (2×10^5 cells) was mixed with VB-48, PI and AO (20, 25 and 0.06 µg/mL, respectively). VB-48 fluorescence was detected using peak excitation at 365 nm and emission at 470/55 nm, AO at 475 nm and emission at 560/35 nm and PI at 530 nm and emission at 675/75 nm. The mean fluorescence intensity (MFI) was calculated from the total fluorescence intensity detected from viable cells and divided by the number of cells.

Mitochondrial Membrane Potential (MMP) Analysis with JC-1

JC-1 has a monomer (green) and aggregate (red) state for ratiometric and semi-quantitative assessment of MMP. The red-to-green fluorescence ratio determines the amount of JC-1 inside mitochondria in relation to the cytosolic amount, serving as a control for changes in cellular dye loading (Perry et al. 2011). HDFs (5×10^5 cells/mL) were incubated with 2.5 µg/mL JC-1 for 10 min at 37°C. Then, HDFs were washed twice with PBS and mixed with DAPI. To determine the maximal MMP depolarization and the limits of the polarized/depolarized gates, an H₂O₂-untreated sample was incubated with CCCP (50 µM) together with JC-1. After exclusion of non-viable cells by DAPI staining, two gates were defined as cells with mostly polarized mitochondria and cells with mostly depolarized mitochondria. These gates correspond to the upper-left gate and lower-right gate, respectively, as shown in Fig. S2. Different incubation times were used for both JC-1 and CCCP to determine that the optimal was 10 min at 37°C. DAPI fluorescence was detected using peak excitation at 365 nm and emission at 470/55 nm, JC-1 monomers at 475 nm and emission at 560/35 nm and JC-1 aggregates at 530 nm and emission at 675/75 nm.

Measurement of Mitochondrial Superoxide Levels (MSLs) with MitoSOX

HDFs (3×10^5 cells/well) were incubated with 5 µM MitoSOX in HBSS for 20 min at 37°C. Then, HDFs were trypsinized and incubated with Hoechst (10 µg/mL) for 15 min at 37°C. Next, HDFs were stained with the non-viable cell staining RedDot2 (Biotium, Hayward, CA) diluted 1,000 times (concentration not specified). An H₂O₂-untreated sample was incubated with 150 µM antimycin for 5 min prior to MitoSOX staining, as a positive control for MitoSOX labelling. The Hoechst fluorescence was detected using peak excitation at 365 nm and emission at 470/55 nm, MitoSOX at 475 nm and emission at 675/75 nm and RedDot2 at 630 nm and emission at 740/60 nm. The mean fluorescence intensity (MFI) was calculated from the total fluorescence intensity detected from viable cells and divided by the number of cells.

Confocal Laser Scanning Microscope (CLSM)

The distribution pattern of MitoSOX fluorescence was analysed by using CLSM 710 (Zeiss, Jena, Germany) using a 63× oil-immersion objective with a numerical aperture of 1.4. MitoSOX fluorescence was detected using the 514-nm line of the multiline argon laser. HDFs were seeded at 50%

confluence in 10-cm² slide flasks (Nunc, Roskilde, Denmark), 24 h before the analysis. HDFs were prepared like for MSL measurement and maintained at 37°C in HBSS during imaging.

Statistical Analysis

The data were subjected to a two-tailed *t*-test analysis and a *P* value less than 0.05 was considered significant (* < 0.05; ** < 0.01; *** < 0.001). The outliers were removed based on the interquartile range. One value is considered an outlier if it is higher than the sum of the third quartile and 1.5 times the interquartile range or it is below than the sum of the first quartile and 1.5 times the interquartile range. The statistical analysis was done using Microsoft Office Excel 2007 software.

The intraassay variation was defined as the variation in the results obtained when the same cellular suspension is measured three times in the image cytometer. The interassay variation was defined as the variation in the results obtained from three independent experiments, where the cell culture was performed in three different days from different starting material. To analyse the intraassay and interassay variability of the cell viability, TRS and MMP, we calculated the coefficient of variation (CV) defined as the ratio of the standard deviation to the mean. On the other hand, to analyse the biological differences between samples, we calculated the standard error of the mean (SEM) defined as the standard deviation divided by the square root of the sample size.

Results

This study describes a protocol for cell phenotyping by studying cellular and mitochondrial functional parameters in human dermal fibroblasts (HDFs) using image cytometry (ICM). To evaluate the protocol, HDFs from healthy individuals were analysed after treatment with 0, 2 and 4 mmol/L H₂O₂ for 1, 1½ and 2 h (Fig. 1). At these time points, we measured changes in four parameters: cell number and viability, thiol redox status (TRS), mitochondrial membrane potential (MMP) and mitochondrial superoxide levels (MSLs). The first three assays were performed with one cell sample leading to reduction in analytical variability, biological material and time (Fig. 1a). MSL was measured in a parallel cell culture (Fig. 1b).

Analysis of the Intraassay and Interassay Variation

A statistical evaluation of the intraassay and interassay variation associated with our protocol is shown in Table 1.

The intraassay variation was calculated from three repeated measurements of the same sample. The coefficient of variation (CV) percentage was different in each parameter: TRS had the highest average CV, MMP the second and cell viability the lowest (12.2, 10.3 and 4.2%, respectively). The CV percentage, in the three parameters, was lower in H₂O₂-untreated samples than in the H₂O₂-treated ones. The interassay variation, caused by cell culture, was studied in four independent experiments performed with one cell line, NHDF-02. In contrast to the test of the intraassay variation, MMP had the highest average CV (56.1%) and TRS had the second highest average CV (18.4%). As in the intraassay variation, cell viability and H₂O₂-untreated showed the lowest CV. In both cases, the CV increased with the H₂O₂ concentration and incubation time. Thus, the protocol should be used carefully at 1.5 and 2 h.

Cell Number and Viability Analysis

Determining the number of cells in culture is important for standardization of culture conditions and to evaluate cell proliferation and cell death (Phelan 1998; Bie et al. 2011). Treatment with H₂O₂ led to a decrease in cell number in a time- and concentration-dependent manner (data not shown). The CV of the cell number was 15% (data from seven independent experiments with two NHDF cell lines) (data not shown). Treatment with H₂O₂ also led to decreased cell viability, in a time- and concentration-dependent manner (Fig. 2a). Incubation periods shorter than 1 h did not show decreased cell viability (data not shown). The maximal decrease occurred after treatment with 4 mmol/L H₂O₂ for 2 h (Fig. 2a).

Thiol Redox Status (TRS) Study

TRS is used to determine the cellular level of free thiols that correlates with the cellular redox state (Skindersoe et al. 2012). Only minimal effects on TRS were observed after more than 2 h of incubation (data not shown). The treatment with H₂O₂ samples resulted in decreased TRS in a concentration- and time-dependent manner (Fig. 2b). The decrease of TRS and the loss of cell viability showed a linear correlation ($R^2 = 0.90$) (Fig. S1).

Mitochondrial Membrane Potential (MMP) Analysis

MMP is an important feature of mitochondria, because it plays a central role in the oxidative phosphorylation and mitochondrial apoptosis regulation, among other pathways (Brand and Nicholls 2011). The H₂O₂-untreated samples showed MMP depolarization of 10–20% of the cell population (Fig. 2c). Cells treated with 2 mmol/L H₂O₂

A)

B)

Fig. 1 Flow chart of phenotyping protocol. **(a)** Approximately 1.6×10^6 HDFs were seeded in a standard T75 cell culture flask. After 24 h, HDFs were treated with 0, 2 and 4 mmol/L H_2O_2 for 1, 1½ and 2 h. Then, HDFs were harvested by trypsinization and collected in a 15-mL Falcon. Four aliquots were taken from the Falcon for cell number, cell viability, thiol redox status (TRS) and mitochondrial

membrane potential (MMP). **(b)** Approximately 3×10^5 HDFs were seeded in a standard p6-well plate. After 24 h, HDFs were treated with 0, 2 and 4 mmol/L H_2O_2 for 1, 1½ and 2 h. Then, HDFs were incubated with MitoSOX for 20 min. HDFs were harvested by trypsinization and collected in a 15-mL Falcon. Hoechst was added to the cell suspension to stain the nucleus of each cell

Table 1 Statistical analysis of the intraassay and interassay variation using the NHDF-02 control cell line

		Viability %			TRS fluorescence intensity (a.u)			MMP polarized %		
		Mean	Intraassay CV %	Interassay CV %	Mean	Intraassay CV %	Interassay CV %	Mean	Intraassay CV %	Interassay CV %
1 h	0 mmol/L H_2O_2	96.0	1.8	1.1	88.4	7.2	8.7	92.7	1.3	6.5
	2 mmol/L H_2O_2	77.4	3.3	12.3	74.7	3.2	17.9	17.7	32.2	58.7
	4 mmol/L H_2O_2	79.8	1.1	4.5	71.7	4.8	8.0	14.2	11.6	79.7
1.5 h	0 mmol/L H_2O_2	94.7	1.7	1.4	90.1	6.9	7.6	93.3	0.3	5.4
	2 mmol/L H_2O_2	46.2	16.4	38.2	59.5	5.8	12.2	27.5	10.5	75.0
	4 mmol/L H_2O_2	28.8	18.3	31.6	48.7	5.8	33.3	45.3	17.4	88.2
2 h	0 mmol/L H_2O_2	94.4	1.2	1.2	101.1	5.6	9.2	69.6	1.4	56.3
	2 mmol/L H_2O_2	35.0	14.2	47.2	51.3	33.7	31.1	41.5	10.6	57.8
	4 mmol/L H_2O_2	29.2	43.4	91.2	31.1	36.6	37.8	41.4	7.2	77.2

The intraassay variation was analysed in four independent experiments with three repeated measurements for each value. The interassay variation was studied by comparison of four independent experiments of the same cell line

for 1 h showed depolarized MMP in 81% of the cell population (Fig. 2c). There was no further decrease in MMP at 4 mmol/L H_2O_2 at any incubation times used. MMP depolarization decreased already after treatment with

2 mmol/L H_2O_2 for 30 min (Fig. S2) while TRS and cell viability showed no decrease (data not shown). These results indicated that MMP was the first parameter to change after H_2O_2 treatment.

Fig. 2 Effect of exogenous H_2O_2 treatment for different time periods in two different NHDF cell lines. NHDFs were treated with 0, 2 or 4 mmol/L H_2O_2 for 1, 1½ and 2 h. **(a)** Viability quantification. Cell viability was quantified by counting DAPI-positive cells in the total cell population. **(b)** Thiol redox status (TRS). HDFs were incubated with VB-48, AO and PI. VB-48 was used to determine the levels of free thiols, AO as a nuclear staining and PI to label dead cells (removed from the analysis). The values shown correspond to the mean fluorescence intensity (MFI). **(c)** Mitochondrial membrane potential (MMP). HDFs were incubated with JC-1 to establish the percentage of cells with a depolarized MMP, and dead cells were

excluded by DAPI staining. **(d)** Mitochondrial superoxide levels (MSLs) were measured by quantification of MitoSOX fluorescence intensity levels after treatment with H_2O_2 . MitoSOX exhibits red fluorescence upon oxidation by superoxide anion in the mitochondrial matrix. Hoechst was used as a nuclear staining and RedDot2 to exclude non-viable cells. The values shown correspond to the mean fluorescence intensity (MFI). The columns represent the mean $\pm$ standard error of the mean (SEM). The data correspond to the mean values of at least three independent experiments for each cell line (NHDF-01 and NHDF-02). Two-tailed *t*-test analysis with **P* value <0.05; ***P* value <0.01; ****P* value <0.001

Mitochondrial Superoxide Level (MSL) Analysis

MSL was measured with MitoSOX, a probe that accumulates in the mitochondria of living cells and fluoresces red when it is oxidized by the superoxide anion (Robinson et al. 2007). We observed increased MSL in a time- and concentration-dependent manner (Fig. 2d). At high concentrations, MitoSOX can stain other organelles besides mitochondria, like the nucleus (Dingley et al. 2011). To ensure that our results reflect only mitochondrial superoxide, we performed cell-imaging experiments using the same MitoSOX incubation conditions. MitoSOX red fluorescence showed a characteristic mitochondrial pattern with no

cytoplasmic background and minimal nucleus staining (Fig. S3).

Analysis of HDFs from Very Long-Chain Acyl-CoA Dehydrogenase Deficiency (VLCADD) Patients

HDFs from VLCADD patients were used to evaluate the capacity of this protocol to phenotype cellular and mitochondrial effects of mutations in the *ACADVL* gene. Based on the high interassay variation of the H_2O_2 treatment at 1.5 and 2 h, we treated the cells only for 1 h. We observed differences between HDFs from the healthy individuals and from the patients and also between the severe (P2) and the

Fig. 3 Analysis of HDFs derived from VLCADD patients after treatment for 1 h with 0 mmol/L, 2 mmol/L or 4 mmol/L of H_2O_2 , respectively. **(a)** Viability quantification. Cell viability was quantified by counting DAPI-positive cells in the total cell population. **(b)** Thiol redox status (TRS). HDFs were incubated with VB-48 for levels of free thiols, AO as a nuclear staining and PI to stain non-viable cells and exclude them from the analysis. The values shown correspond to the mean fluorescence intensity (MFI). **(c)** Mitochondrial membrane potential (MMP). HDFs were incubated with JC-1 to establish the percentage of cells with a depolarized MMP and with DAPI to

exclude non-viable cells. **(d)** Mitochondrial superoxide levels (MSLs) were measured by quantification of MitoSOX fluorescence intensity. Hoechst was used as a nuclear staining and RedDot2 as a non-viable cell staining to exclude non-viable cells. The values shown correspond to the mean fluorescence intensity (MFI). The columns represent the mean $\pm$ standard error of the mean. The data shown correspond to at least three independent experiments for each cell line, two NHDFs (NHDF-01 and NHDF-02) and two HDFs from VLCADD patients (P1 and P2). Two-tailed *t*-test analysis with **P* value <0.05 ; ***P* value <0.01 ; ****P* value <0.001

mild (P1) patient (Fig. 3). Cell viability analysis showed statistically significant but very small increase in the H_2O_2 -untreated HDFs from the mild VLCADD (P1). There were no differences in viability between patients and healthy individuals at 2 mmol/L H_2O_2 , but HDFs from both VLCADD patients showed significantly decreased viability at 4 mmol/L H_2O_2 (Fig. 3a). TRS showed no significant differences between any HDFs from compared to HDFs from healthy individuals (Fig. 3b). MMP showed no differences in the H_2O_2 -untreated samples and decreased with the H_2O_2 treatment, being lower in HDFs from mild VLCADD (P1) than in HDFs from severe VLCADD (P2) (Fig. 3c). MSL was significantly increased in the H_2O_2 -untreated samples that further increases in the H_2O_2 -treated samples with the highest levels in HDFs from the severe patient (P2) (Fig. 3d and Fig. S4).

Discussion

In this paper, we present a protocol for assessing cellular and mitochondrial parameters in human dermal fibroblasts (HDFs) by image cytometry (ICM). Using our protocol, we measured cell number, viability, TRS and MMP in one sample, reducing biological material, time and analytical variability. As a supplement, MSL was analysed in a parallel experiment (Fig. 1). The different parameters responded to induced oxidative stress by H_2O_2 in HDFs from healthy individuals in a concentration- and time-dependent manner. Furthermore, we successfully detected differences between HDFs from patients with VLCADD and healthy individuals, showing the capacity of this protocol to phenotype consequences of genetic changes in inherited diseases.

Cell viability is used to evaluate the amount of living cells in a cell population under treatments or to determine whether the expression of a mutation affects cell proliferation or promotes cell death (Phelan 1998; Bie et al. 2011). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) and similar assays have been widely used to determine cell viability or cell proliferation. The results of these assays are ambiguous because one cannot distinguish between changes in average enzyme activity and changes in the number of cells (Halter 2012). Our protocol for cellular phenotyping overcomes these problems because it analyses single cells (Fig. 2a). While cell count and viability are a measure of living and dead cells, parameters like MMP and TRS can offer additional information about cellular physiology (Fig. 2). MMP is a key parameter for mitochondrial function (Brand and Nicholls 2011; Cottet-Rousselle et al. 2011). In our study, MMP showed higher sensitivity to the H₂O₂ treatment than viability and TRS, allowing us to assess effects on cellular physiology at an early stage and supporting our hypothesis that mitochondrial dysfunction is an early marker of cellular stress (Fig. 2c and Fig. S2). Combining TRS and MMP gives complementary information because MMP is capable of detecting mitochondrial dysfunction while TRS reveals the status of the cellular redox balance.

Eukaryotic cells have a reducing inner environment that switches to an oxidizing environment upon oxidative stress conditions. Cellular redox homeostasis depends mostly on the size of the pool of reduced glutathione in the cytoplasm, a main cellular antioxidant whose free thiol group can act as a ROS scavenger (Skindersoe et al. 2012). Therefore, TRS is an informative parameter of the redox balance and an indirect parameter of intracellular ROS levels (Fig. 2b). Treatment with H₂O₂ increased MSL and decreased TRS but not in a linear correlation (Fig. 2b, d). This might suggest that there is a cross talk between these parameters, but other players may also be involved.

Statistical evaluation of the intraassay variation of our protocol showed that TRS had the highest CV, most likely because it was not scored as a discrete but a continuous parameter (Table 1). Evaluation of the interassay variation showed that MMP had the highest variation consistent with the notion that this is the most sensitive parameter. At the highest H₂O₂ concentration and longest treatment times, the variation was very high; thus, we suggest that 1-h treatment should be preferentially used.

We used HDFs from patients with VLCADD. VLCAD is a mitochondrial enzyme that catalyses the first step in the β -oxidation of fatty acids with a chain length of 14–20 carbons. Defects in VLCAD lead to impaired fatty acid β -oxidation and have been implicated with mitochondrial dysfunction as well as increased ROS levels (Tucci et al. 2010; Cardoso et al. 2013). VLCADD patients show

different clinical phenotypes that might correlate to specific patterns of cellular phenotypes; thus, we chose both a patient with mild and a patient with severe phenotype. Indeed, already in the H₂O₂-untreated samples, we observed some differences: MSL was highest in HDFs from the severely affected VLCADD patient (P2), lower in the mild (P1) and significantly lower in the controls (Fig. 3d). The increased MSL might be linked to the observed tendency of higher TRS. The latter could be caused by a higher expression of the cellular redox markers like glutathione in response to a chronic state of oxidative stress.

An induction of survival mechanisms in the H₂O₂-untreated conditions could be caused by oxidative stress, as shown by increased MSL and cellular viability (Fig. 3a, d). Because ROS can both act as signalling molecules and as damaging species, the balance between signalling and damaging is mostly concentration dependent (Valko et al. 2006; Murphy 2009). Indeed, at 2 mmol/L and 4 mmol/L H₂O₂, we observed a different behaviour between HDFs from mild VLCADD (P1) and from severe VLCADD (P2) in all the parameters (Fig. 3 and Fig. S4b). This supports our idea of using an oxidative stressor like H₂O₂ to evaluate differences between milder and severe phenotypes.

Under oxidative stress, HDFs from the severe patient (P2), but not the mild (P1), retained a higher population of functional mitochondria measured by MMP (Fig. 3c). It has been hypothesized that VLCAD is a stabilizing factor for the supercomplexes of the respiratory chain and that absence of VLCAD protein might destabilize them (Wang et al. 2010). The HDFs from the severe VLCADD patient (P2) express mRNA with premature stop codon that either is degraded by nonsense-mediated decay (NMD) or leads to very low levels of truncated VLCAD protein. The HDFs from the severe VLCADD patient (P2) express mRNA with a premature stop codon that either is degraded by nonsense-mediated decay (NMD) or leads to very low levels of truncated VLCAD protein. This correlates with the clinical symptoms showed by the patient; a severe phenotype is caused by the lack of VLCAD enzyme activity. However, MMP in HDFs from the severe patient (P2) and from healthy individuals did not differ significantly under any of the conditions studied. Surprisingly, HDFs from the mild VLCADD patient (P1) displayed a significant disruption of the MMP after treatment with H₂O₂ (Fig. 3c), although this patient shows clinical symptoms that correspond to a mild phenotype. These clinical symptoms correlate with the genotype of the mild patient (P1) that harbours a missense mutation (p.Thr230Ile) that potentially can partially express both functional folded and misfolded VLCAD protein. Western blotting of protein lysates from HDFs from the mild patient (P1) indeed shows residual VLCAD protein (unpublished data) that could correspond to both some

folded and some misfolded VLCAD protein, the latter of which could interact incorrectly with the supercomplexes thus leading to a disruption of MMP.

In summary, our results from the combined analysis of cellular viability, TRS, MMP and MSL showed the potential of our protocol for cellular and mitochondrial phenotyping of HDFs from patients with inherited diseases. We observed consistency in repetitive analysis and between the different parameters. The results of this protocol have the potential to distinguish order of events and to pinpoint potential molecular pathological mechanism that then can be studied further. This establishes our protocol as efficient and highly potential for monitoring cellular and mitochondrial function in HDFs using ICM.

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Take-Home Message

Protocol for cellular and mitochondrial phenotyping reveals differences between fibroblasts from VLCADD patients with mild and severe gene variations.

Compliance with Ethical Guidelines

Conflict of Interest

Paula Fernandez Guerra, Martin Lund, Thomas Juhl Corydon, Nanna Cornelius, Niels Gregersen, Johan Palmfeldt and Peter Bross declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5).

Details of the Contributions of Individual Authors

1. Paula Fernandez Guerra: experimental design, performance of experiments with fibroblasts from healthy individuals, data analysis and writing the first draft of the manuscript
2. Martin Lund: performance of experiments with fibroblasts from patients

3. Thomas Juhl Corydon: performance of confocal laser microscope experiments with MitoSOX
4. Nanna Cornelius: performance of confocal laser microscope experiments with MitoSOX
5. Niels Gregersen: VLCADD patient selection and hypothesis of oxidative stress in VLCADD patients
6. Johan Palmfeldt: experimental design, report editing and data analysis
7. Peter Bross: experimental design, report editing and data analysis

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