Supplemental Data

Altered Redox Mitochondrial Biology in the Neurodegenerative Disorder Fragile X-Tremor/Ataxia Syndrome: Use of Antioxidants in Precision Medicine

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SUPPLEMENTARY METHODS

Electron Paramagnetic Resonance (EPR)

Direct detection of some free radicals is very difficult due to their relatively low concentrations (<nmol/L), high reactivity and short half-life. To evaluate and identify oxygen-centered free radicals in control and premutation fibroblasts, we utilized the spin trapping technique, in which the target free radical reacts with a spin trap (in our case 5,5′-dimethyl-1-pyrroline-N-oxide or DMPO). DMPO is used for the detection of several types of radicals (1), among them superoxide anion and hydroxyl radicals, which will react with the compound, giving stable EPR spectra (2) (Supplementary Figure S1).

For optimization purposes we run a positive control consisting of a reaction with xanthine oxidase and hypoxanthine in PBS (pH 7.4) supplemented with deferoxamine mesylate (iron chelator) and DMSO. The EPR spectrum obtained with the positive control consisted of three adducts between DMPO and the following radicals: superoxide anion, hydroxyl and methyl radicals at a ratio of 15:9:1 ($a_N = 14.9$ G for DMPO-hydroxyl; $a_N = 16.4$ G and $a_{1H}$ = 23.3 G for DMPO-methyl). For further details refer to the Materials and Methods and Results sections of the main text.

Complex I, Complex IV and Citrate Synthase Activities

The activity of NADH-quinone oxidoreductase (NQR, Complex I) was evaluated at 340 nm following the oxidation of NADH at 37°C as previously described (3). In 160 μL of water, 5 μg of protein were added, mixed and incubated for 2 min at 37°C. Then, 50 μL of buffer containing 5 mg/mL BSA, 240 μmol/L KCN, 4 μmol/L antimycin A, 40 mmol/L HEPES/KOH, pH 7.5, with or without 5 μmol/L rotenone, were added. The reaction was started with the addition of 50 μmol/L 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinone. The absorbance changes were followed in a Tecan Infinite M200 microplate reader equipped with the Magellan software for 10 min. Rotenone-sensitive activities were calculated from the linear part of ΔA versus time plots and using an extinction coefficient of 6.22 (mM × cm)^−1.

The activity of Complex IV was measured as previously described (3,4). Briefly, the oxidation of reduced cytochrome c was followed at 550 nm and 37°C in the presence of 3–5 μg of protein in a buffer containing 0.25 mol/L sucrose, 1 mg/mL BSA, 10 mmol/L KH₂PO₄, pH 6.5, upon addition of 2.5 mmol/L lauryl maltoside, with or without 240 μmol/L KCN. The reaction was started with the addition of 10 μmol/L reduced cytochrome c. The absorbance changes were followed for 5 min in a Tecan Infinite M200 microplate reader equipped with the Magellan software. Activity was calculated from the linear part of LN absorbance versus time plots and using an extinction coefficient of 21 (mmol/L × cm)^−1.

The activity of citrate synthase was evaluated in fibroblasts at 30°C and 412 nm following the reduction of 0.1 mmol/L 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of 2–8 μg of cell lysate, 0.2 mmol/L acetyl-CoA in a medium with 10 mmol/L Tris-HCl, pH 8.1 and 0.2% Triton X-100. The reaction was started by adding 0.5 mmol/L oxalacetate acid. The absorbance changes were followed in a Tecan Infinite M200 microplate reader equipped with the Magellan software. Rates were calculated from the linear part of delta Abs/min and expressed by mg protein, using an extinction coefficient of 11,400 (mol/L × cm)^−1.
Genomic DNA was first isolated from $1 \times 10^6$ fibroblasts using the Gentra Puregene Cell and Tissue Kit (Qiagen) following the manufacturer’s recommendations for cells. Concentration and purity of DNA was measured at an absorbance of 260 nm and 280 nm using the Tecan i-control 1.6 software (v. 1.6.19.2) on a Tecan infinite M200 Nanoquant. DNA yield was 5–10 μg, and DNA purity was ≥1.85. DNA stock was diluted to 0.626 ng/μL with nuclease-free water and served as stock for real-time PCR (qPCR). Following DNA extraction, changes in mtDNA copy number and gene deletions were evaluated by qPCR using dual labeled probes as previously described (5,6). The targeted genes were single-copy nuclear PK (Pyruvate kinase) and mitochondrial NDI (NADH oxidase).
Dehydrogenase Subunit I) and ND4 (NADH Dehydrogenase Subunit IV). Species-specific primers were selected using the Primer Express 3 software (Applied Biosystems). Because minor changes in the efficiency of the amplification of each gene can result in large changes in the ratio of mtDNA/nDNA, the mitochondrial primers were designed for regions of low polymorphisms (less than 1%). Human primers for PK were: forward 5'-AGCCCA AAATG GCTCT GAAG-3'; reverse 5'-AGAGA CAGAA TGCCA GTGAG CT-3'. The probe used was Roche UPL library #11, Locked Nucleic Acids, short hydrolysis probes, labeled at the 5' end with fluorescein and at the 3' end with a dark quencher dye. ND1 primers and probe were: forward 3485-3504, 5'-CCCTA AACC CGCCA CATCT-3'; reverse 3532-3553, 5'-GAGCG ATGGT GAGAG CTAA GT-3'; probe 3506-3529, 5' FAM-CCATC ACCCT ATCA TCACC GCCC-BHQ3'. ND4 primers and probe were the following: forward 12087-12109, 5'-CCATT CTCTT CCTAT CCCCT AAC-3'; reverse 12140-12170, 5' CACAA TCTGA TGTTT TGTTT AAACCT ATATT T-3'; probe 12111-12138, 5'FAM-CCGAC ATCAT TACCC GTTTT CCTCT TG-3'. QPCR was performed on a Mastercycler EP Realplex thermocycler (Eppendorf). QPCR was performed in a 96-well PCR plate with TaqMan Universal PCR Mastermix (Applied Biosystems) with 400 nmol/L of each primer, 80 nmol/L of fluorescent probe and 5 μL of 3.13 ng total of template DNA per reaction. Amplification was performed using the default cycling parameters of 2 min at 50°C (activation of UNG), 10 min at 95°C (AmpliTaq Gold activation), followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The mean cycle time obtained by double derivative (CalqPlex algorithm; Eppendorf) was designated as Ct. Relative mtDNA copy number was assessed by a comparative Ct method using the following equation: mtDNA/nDNA = 2−ΔCt, where ΔCt = Ct ND1 − Ct PK. Deletions in ND4 were determined using the following equation: 2−ΔCt, where Ct ND4 − Ct ND1. Please see supplementary material for details on our qPCR method.

Metabolomics of Cultured Fibroblasts

The metabolome is the entire collection of metabolites in a biological system, such as cells and tissues. Untargeted metabolomics refers to the global relative quantitative analysis of the metabolome. Untargeted metabolomics allows the analysis of hundreds to thousands of individual metabolites. Such detailed metabolomic profiles can be the most direct reflection of the current state of a biological system and are the basis for the concept of personalized medicine.

Thirty μL aliquots were extracted by 1 mL of degassed acetonitrile: isopropanol: water (3:3:2, V/V/V) at –20°C, centrifuged and decanted with subsequent evaporation of the solvent to complete dryness. A clean-up step with acetoni-trile/water (1:1) removed membrane lipids and triglycerides. The cleaned extract was aliquoted into two equal portions and the supernatant was dried down again. Internal standards C08-C30 FAMEs were added and the sample was derivatized by methoxyamine hydrochloride in pyridine and subsequently by N-methyl-N-trimethylsilyltrifluoroacetamide for trimethylsilylation of acidic protons. Data acquisition chromatographic parameters were acquired using the following chromatographic parameters: Column: Restek corporation rtx5sil-MS (30 m length × 0.25 mm internal diameter with 0.25 μm film made of 95% dimethly/5% diphenylylo-siloxane); Mobile phase: He; Column temperature: 50–330°C; Flow-rate: 1 mL × min−1; Injection volume: 0.5 μL; Injection: 25 splitless time into a multi-baffled glass liner; Injection temperature: 50°C ramped to 250°C by 12°C s−1; Gradient: 50°C for 1 min, then ramped at 20°C min−1 to 330°C, held constant for 5 min. Mass spectrometry parameters were used as follows: a Leco Pegasus IV mass spectrometer with unit mass resolution at 17 spectra s−1 from 80–500 Da at ~70 eV ionization energy and 1,800 V detector voltage with a 230°C transfer line and a 250°C ion source. Data processing: Raw data files were preprocessed directly after data acquisition and stored as ChromaTOF-specific *.txt files, as generic *.txt result files and additionally as generic ANDI MS *.cdf files. ChromaTOF versus 2.32 was used for data pre-processing without smoothing, 3 s peak width, baseline subtraction just above the noise level, and automatic mass spectral deconvolution and peak detection at signal/noise levels of 5:1 throughout the chromatogram. Apex masses were reported for use in the BinBase algorithm. Result *.txt files were exported to a data server with absolute spectra intensities and further processed by a filtering algorithm implemented in the metabolomics BinBase database. The BinBase algorithm used the following settings: validity of chromatogram (<10 peaks with intensity >107 counts s−1), unbiased retention index marker detection (MS similarity >800, validity of intensity range for high m/z marker ions), retention index calculation by fifth-order polynomial regression. Spectra are cut to 5% base peak abundance and matched to database entries from most to least abundant spectra using the following matching filters: retention index window ± 2,000 units (equivalent to about ± 2 s retention time), validation of unique ions and apex masses (unique ion must be included in apexing masses and present at >3% of base peak abundance), mass spectrum similarity must fit criteria dependent on peak purity and signal/noise ratios and a final isomer filter. Failed spectra were automatically entered as new database entries.
entries if s/n > 25, purity < 1.0 and presence in the biological study design class was >80%. All thresholds reflected settings for ChromaTOF versus 2.32. Quantification was reported as peak height using the unique ion as default, unless a different quantification ion is manually set in the BinBase administration software BinView. A quantification report table was produced for all database entries that were positively detected in more than 10% of the samples of a study design class (as defined in the miniX database) for unidentified metabolites. A subsequent post-processing module is employed to automatically replace missing values from the *.cdf files. Replaced values were labeled as low confidence by color coding, and for each metabolite, the number of high-confidence peak detections was recorded as well as the ratio of the average height of replaced values to high-confidence peak detections. These ratios and numbers were used for manual curation of automatic report data sets to data sets released for submission.

Metabolite identification was performed through the use of several databases including PubChem Compound (7), KEGG (8) and HMDB (9–11) and the online chemical translation service (12). Univariate method (performed with MetaboAnalyst and XLSTAT) was used to analyze and visualize the data because provides a preliminary overview about features that are potentially significant in discriminating the conditions under study. For paired fold-change analysis, the algorithm first counts the total number of pairs with fold changes that are consistently above/below the specified LOG2 FC threshold for each variable (± 0.3). A variable is reported as significant if this number is above a given count threshold (>75% of pairs/variable). The purpose of fold change is to compare absolute value changes between the two diagnostic group means. Data were used before normalization and the results were plotted as in LOG2 scale, so that equal fold changes (up/downregulated) will have the same distance to the zero baseline.

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