Analysis of heat-induced protein aggregation in human mitochondria

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Supporting experimental procedures

Peptide preparation and MALDI–TOF analysis

Manually exised protein spots were washed and destained stepwise with water, 50% acetonitrile (ACN) and 100% ACN. Gel pieces were dried in a vacuum concentrator (ScanVac, Labogene) for 30 min at 40°C and 1000 x*g* and afterwards stepwise reduced with 20 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate for 30 min at 55°C and alkylated with 40 mM iodacetamide (IAA) in 50 mM ammonium bicarbonate for 30 min at room temperature in the dark. After washing, the gel pieces were dehydrated with 100% ACN and dried in a vacuum concentrator. Proteins were digested with sequencing grade trypsin (400 pg/gel piece in 50 mM ammonium bicarbonate) at 37°C over night. The peptide extract was collected and the remaining peptides were extracted with 50% ACN. The peptides were dried in a vacuum concentrator and stored at –20°C. Peptides were dissolved in 8 µl 0.1% TFA. 1 µl was dried on an AnchorChip 384 target plate (Bruker Daltonik GmbH, Bremen, Germany). Matrix solvent (85% acetonitrile, 0.1% TFA, 1 mM $NH_4H_2PO_4$) was saturated with α-Cyano-4-hydroxycinnamic acid at room temperature. The solution was diluted 1:30 with matrix solvent and 1 µl of the diluted matrix solution was added to the dried peptides.

MALDI MS and MS/MS measurements were run with an automated method on a Bruker Autoflex III spectrometer in positive reflectron mode. 4000 MS1 spectra were recorded (700–3150 m/z), processed by FlexAnalysis 3.3 software and sent to ProteinScape 3.0 for evaluation. Suitable peptide ions were subjected to MS/MS analyses (LIFT-process without collision gas) with acquisition of 10000 spectra per precursor. Peptide identification was done with an in house Mascot server version 2.4 (Matrix Science Ltd, London, UK) searching human sequences in the SwissProt database. Oxidation of methionine and carbamidomethyl on cysteine were set as variable modification. Identifications that were only based on MS1 data (peptide mass fingerprint) were inspected manually for number of identified peptide peaks, completeness of assignment, and mass errors.

Immunocytochemistry

For fluorescence microscopy 3 x 10^4 cells per well were grown over night on coverslips. For heat shock, culture medium was changed with 45°C preheated medium and cells were incubated in an incubator at 45°C for 2 h. After heat treatment cells were incubated for 5 min at 37°C in fixing solution (4% (w/v) paraformaldehyde, 10% (w/v) sucrose in 1x PBS, pH 7.2) and permeabilized with 0.5% triton X-100 in PBS for 10 min at RT. Cover slips were washed three times in PBS after each step. After 1 h incubation in blocking solution (0.2% (w/v)

bovine serum albumin (BSA) in PBS) samples were incubated over night with different primary antibodies (1:250 dilution in blocking solution). Flourophore-coupled secondary antibodies Alexa Fluor 488 and 594 (Invitrogen) were used to generate fluorescence signals. Microscopic pictures were obtained using $EVOS_{FI}$ Cell Imaging System (Peqlab) with a 40x objective lens.

Sucrose density gradient centrifugation

300 µg of fresh isolated mitochondria were resuspended in 1000 µl resuspension buffer (500 mM sucrose, 160 mM KAc, 40 mM HEPES/KOH pH 7.6, 10 mM MgAc, 5 mM glutamate, 5 mM malate, 1 mM DTT) and incubated at 25°C, 37°C, 42°C and 45°C for 20 min. Mitochondria were reisolated (12,000 x*g*, 10 min, 4°C), resuspended in gradient buffer (0.6 M sorbitol, 10 mM MOPS/KOH pH 7.2, 2 mM PMSF) and lysed with1% digitonin. Samples were loaded on a 15–65% sucrose gradient in gradient buffer and separated at 200,000 x*g* for 1 h, 4°C. Fractions (1–23) of 500 µl each were diluted 1:1 in gradient buffer and precipitated with TCA. Samples were analyzed by SDS–PAGE and Western blot.

Influence of high salt concentrations on the Tufm aggregation

30 µg of fresh isolated mitochondria were resuspended in 100 µl resuspension buffer (500 mM sucrose, 160 mM KAc, 40 mM HEPES/KOH pH 7.6, 10 mM MgAc, 5 mM glutamate, 5 mM malate, 1 mM DTT) and heat stressed as described before (42°C, 20 min). After isolation of the aggregates (125,000 x*g*), supernatants (Sup 1) were precipitated by TCA and pellets were reextract in resuspension buffer containing 0.2 M, 0.5 M or 1 M KCl. Soluble proteins (Sup. 2) and unsoluble proteins were separated again by centrifugation and samples were analyzed by SDS–PAGE and Western blot.

Recovery of *in organello* translation after heat shock

Intact energized mitochondria were heat stress for 20 min as described before and up to 2 h incubated at 25°C (recovery). The amount of newly synthesized mitochondrial proteins was tested *in organello* as described before.

Mitophagy assay

The induction of mitophagy due to stress was determined by incubation of cultured cells either for 1 h at 45 °C or for 30 min in presence of menadione (final concentration (f. c.) 0.5 mM). Half of the samples were incubated for 8 h at 37 °C for recovery. As control for Pink1 (PTEN-induced putative kinase 1) induction a sample was treated with carbonyl cyanide m-chlorophenyl hydrazine (CCCP; f. c. 10 µM) for 9 h. Cells were lysed in lysis buffer (0.5% (v/v) Triton X-100, 200 mM KCl, 30 mM Tris/HCl pH 7.4, 5 mM EDTA, 0.5 mM PMSF, 1x protease inhibitors) by vigorous shaking for 10 min at 4°C. and analyzed by SDS-PAGE and Western blot.