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Research paper

Effect of Lon protease knockdown on mitochondrial function in HeLa cells

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ABSTRACT

ATP-dependent proteases are currently emerging as key regulators of mitochondrial functions. Among these proteolytic systems, Lon protease is involved in the control of selective protein turnover in the mitochondrial matrix. In the absence of Lon, yeast cells have been shown to accumulate electron-dense inclusion bodies in the matrix space, to loose integrity of mitochondrial genome and to be respiratory deficient. In order to address the role of Lon in mitochondrial functionality in human cells, we have set up a HeLa cell line stably transfected with a vector expressing a shRNA under the control of a promoter which is inducible with doxycycline. We have demonstrated that reduction of Lon protease results in a mild phenotype in this cell line in contrast with what have been observed in other cell types such as WI-38 fibroblasts. Nevertheless, deficiency in Lon protease led to an increase in ROS production and to an accumulation of carbonylated protein in the mitochondria. Our study suggests that Lon protease has a wide variety of targets and is likely to play different roles depending of the cell type.

1. Introduction

Mitochondria are a major source of intracellular reactive oxygen species (ROS), the production of which increases with ageing and cancer. The deleterious effects of ROS may be responsible for the impairment of mitochondrial function observed during various pathophysiological states associated with oxidative stress, ageing and cancer. These organelles are also targets of oxidative damage (oxidation on mtDNA, lipids, proteins) [1]. An important factor for protein maintenance in the presence of oxidative stress is the enzymatic removal of oxidative modifications and/or protein degradation. Two soluble ATP-dependent proteases, Lon and ClpXP, are found in the matrix of mammalian mitochondria. Lon is a homo-oligomeric complex whereas ClpXP is a hetero-oligomeric complex. This hetero-oligomer of ClpXP is comprised of two subunits: ClpP, the proteolytic component, and ClpX, the ATPase component [2]. Currently, information regarding the role and the identity of specific protein substrates for each of the ATP-dependent proteases is limited [3–7]. The Lon protease is especially efficient in the recognition and proteolysis of misfolded and damaged protein structures [8]. Reduced Lon activity has been associated with insufficient degradation and accumulation of oxidized protein, impaired mitochondrial function and cell death in WI-38 fibroblasts [9]. The Lon protease has been reported to exhibit an age-related impairment in skeletal muscle, liver and heart that was associated with a buildup of oxidatively modified protein [10,11]. Age-dependent increases in the levels of oxidatively modified protein may therefore be due, in part, to decreased clearance of damaged protein by proteases such as Lon. Lon

Abbreviations: ROS, reactive oxygen species; MTT, 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazoliumbromide; COX, cytochrome c oxidase; VO_2, oxygen consumption rate; FCCP, carbonyl cyanide-trifluoromethoxy phenylhydrazine; CCCP, carbonyl cyanide-chloro phenylhydrazine; HBSS, Hank's buffered salt solution.

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protease expression is enhanced in mammalian cells exposed to stresses such as heat shock, oxidative stress, endoplasmic reticulum stress and hypoxia while transient overexpression of Lon protease partially prevents mitochondrial dysfunction [12–15]. In addition to its proteolytic activity, mammalian Lon has also been shown to display chaperone properties and to specifically bind sequences of human mitochondrial DNA and RNA, as well as to interact with mitochondrial DNA polymerase γ, the twinkle helicase and to degrade TFAM [16,17]. Therefore, Lon may participate in replication/transcription of mDNA. Lon is emerging as an important player in the mitochondria by operating in protein quality control and stress response pathways. The effects of its down regulation have been preferentially evaluated in *Escherichia coli*, *Saccharomyces cerevisiae* and *Drosophila melanogaster* [18–20]. Few studies investigated the effects of Lon down-regulation in human cells [9,16,21–23]. We have recently shown that Lon activity declines in old yeast and that Lon deficiency shortens the replicative life span of yeast mother cells [24]. This accelerated ageing of cells disrupted for Lon is accompanied by elevated cytosolic levels of oxidized and aggregated proteins, as well as reduced proteasome activity. In order to address the role of Lon in mitochondrial functionality in human cells, we have set up a HeLa cell line stably transfected with a vector expressing a shRNA under the control of a promoter, which is inducible by Doxycycline. We demonstrated that reduction of Lon protease results in a mild phenotype. Indeed, we have found no change in respiration, as well as in the enzymatic activities of the electron transport system complexes in cell disrupted for Lon. No changes in mitochondrial dynamics and no increase of mitophagy were observed. Although down regulation of Lon protease is not associated with a severe phenotype in HeLa cells, we have found that Lon deficiency leads to an increase ROS production and an increased level of oxidatively modified protein.

2. Material and methods

2.1. Materials and cells

All reagents were of the highest purity and purchased from (Sigma–Aldrich, St. Louis, MO, USA) unless otherwise stated. HeLa T-Rex™ cells were transfected (Invitrogen Life Technologies, Carlsbad, CA, USA) with a 68-base Lon-specific oligonucleotide (5′-gatctccgttcgtctcgcccagccttttcaagagaaaggctgggcga-gacgaacttttttggaaagctt-3′) annealed to its complementary sequence pENTR/H1/T0+ (Block it-Invitrogen) and inserted into pENTR/H1/T0+ (Block it-Invitrogen) as previously reported [25]. Clones of HeLa-tet-on-shLon cells were selected in Dulbecco's Modified Eagle's Medium (Gibco Life Technologies, Carlsbad, CA, USA) 1 g/L glucose supplemented with 10% fetal bovine serum, 50 μg/ml uridine (Invitrogen), 5 μg/ml blasticidin (Invitrogen), and 200 μg/ml zeocin (Invitrogen) and cultured in the presence or absence of 2 μg/ml Doxycycline.

2.2. Cell viability analysis

Cell viability analysis was performed by MTT assay. Cells were stained with MTT reagent (5 mg/mL). After 2 h incubation with MTT reagent, the crystals were dissolved with DMSO. Two hundred microliters of the final solutions were analysed at 562 nm on a spectrophotometer (Fluostar Galaxy; bMG, Stuttgart, Germany).

2.3. RNA isolation and real-time quantitative PCR analysis

Total RNA from cultured cells was extracted using a NucleoSpin® RNA II kit (Macherey Nagel, Düren, Germany). A reverse transcription was performed on 3 μg of total RNA using a SuperScript® III First-Strand Synthesis System (Invitrogen). PCRs were performed on a LightCycler® 480 Real-Time PCR system (Roche Applied Science, Mannheim, Germany) at the technical platform of the IFR 83 at Université Pierre et Marie Curie-Paris 6. Each reaction was carried out in 10 μl with 2 μl of cDNA (equivalent to 10 ng of reverse transcribed RNA), 500 nM primer concentration, and 1X LightCycler® 480 SYBR Green I master mix (Roche Applied Science). Serial dilutions of cDNA (40-2 ng) were used to generate a quantitative PCR standard curve. To normalise gene expression, ACTB was used as a reference gene and was amplified with GCTACGAGCTGCCTGACG as forward primer and GGCTGGAAGAGTGCCTCA as reverse primer. The LightCycler protocol was as follows: 5 min of 95 °C hot start enzyme activation; 45 cycles of 95 °C denaturation for 10 s, 60 °C annealing for 10 s, and 72 °C elongation for 10 s; and melting at 95 °C for 10 s, 65 °C for 60 s, and then heating to 97 °C. Water was used as the template for negative control amplifications included with each PCR run. All reactions were performed in triplicate. Data were analysed using Roche LightCycler® 480 Software, and CP was calculated by the second derivative maximum method. The amount of the target gene mRNA was examined and normalised to the *ACTB* gene mRNA. The relative expression ratio of a target gene was calculated as described [26], based on real-time PCR efficiencies. Primers pairs are listed in Table 1 and were designed using Probe Finder software (available on-line from Roche Applied Science).

2.4. Morphological analyses

For immunofluorescent labelling of the mitochondrial compartment, cells were plated at subconfluence on glass coverslips at least 72 h before their rapid wash in warm PBS followed by fixation in 4% paraformaldehyde and 0.1% glutaraldehyde. Mitochondrial compartment was visualized using polyclonal antibodies against cytochrome c oxidase subunit 2 (anti-COX2) [27]. Coverslips were mounted in Mowiol containing 0.15 μg/mL DAPI. Fluorescent images were acquired as previously described [28].

2.5. mtROS assay

mtROS production was determined using MitoSOX Red mitochondrial superoxide indicator (Invitrogen), which is selectively targeted to the mitochondria and is fluorescent upon ROS oxidation. MitoSOX was used according to the manufacturer's protocol and published literature [29]. Briefly, cells were incubated with MitoSOX (5 μM) in cell culture media. Cells were then pretreated or not with inhibitors (2.5 μg/mL antimycin) as indicated. Fluorescence intensity was recorded immediately after the addition of the probe (t0), and then during a kinetic measurement of 15 min. Fluorescent signal emitted from the oxidized MitoSOXTM Red reagent was detected by flow cytometry (BD Accuri™ C6, BD Biosciences, CA, USA). Rate of superoxide production during the kinetic measurement was quantified by plotting the fluorescent values with time and by calculating the slope of the fitted curve.

2.6. Isolation of mitochondria and preparation of protein extracts

Cells were harvested and resuspended in MST buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris–HCl, pH 7.5). All the subsequent steps were carried out at 4 °C. Cells were homogenized using a dounce. Unbroken cells were removed by centrifugation at 1000 g for 5 min and supernatants were centrifuged (14,000 g, 10 min, 4 °C). Mitochondria pellets were resuspended with MST. Protein content was determined using the Bradford method (Bio-Rad, Marnes la coquette, France). Submitochondrial fractionation and