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Exposure to arginine analog canavanine induces aberrant mitochondrial translation products, mitoribosome stalling, and instability of the mitochondrial proteome

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ABSTRACT

Impairment of mitochondrial protein homeostasis disrupts mitochondrial function and causes human diseases and aging, but the molecular mechanisms of protein synthesis and quality control in mammalian mitochondria are not fully understood. Here we demonstrate in human cells that misincorporation of an arginine analog, canavanine, during mitochondrial protein synthesis, induced aberrant translation products and destabilized the mtDNA-encoded proteome, leading to loss of mitochondrial respiratory chain complexes. Furthermore, in the presence of a high concentration of canavanine, mitoribosome stalling could be demonstrated. The stalling did not, however, occur at arginine codons, but downstream of those codons. In particular, two adjacent arginines induced the most prominent downstream stalling effect, with the distance between the arginine codons and the stalling peak corresponding roughly to the length of the ribosomal exit tunnel. These results suggest that misincorporated canavanine disrupted the proper folding of the hydrophobic nascent polypeptides within the exit tunnel or while being inserted into the inner mitochondrial membrane. The canavanine treatment provides a model system for studying the consequences of mitoribosome stalling and the responses to misfolded proteins exiting the mitochondrial ribosome.

1. Introduction

Protein synthesis within mitochondria is essential for maintaining mitochondrial oxidative phosphorylation (OXPHOS), which produces the majority of cellular ATP energy. Mitochondrial ribosomes synthesize the 13 subunits of OXPHOS complexes, which are encoded by mitochondrial DNA (mtDNA). These subunits are highly hydrophobic and thus directly synthesized to the mitochondrial inner membrane. Since the rest of the OXPHOS subunits are nuclear-encoded and imported into mitochondria from the cytoplasm, the input of proteins synthesized within both compartments is essential for mitochondrial function. Many details of the mitochondrial protein synthesis and its quality control in mammals are not presently known (Hallberg and Larsson, 2014; Lightowlers et al., 2014), but the identification of human diseases that are caused by defects in the mitochondrial tRNAs, aminoacyl-tRNA synthetases (ARSs), translation factors and the mitoribosome has increased the interest to uncover these processes (Abbott et al., 2014; Konovalova and Tyynismaa, 2013; Pearce et al., 2013; Rotig, 2011).

The fidelity of protein synthesis is tightly controlled at the level of tRNA charging by ARSs, which recognize amino acids corresponding to their cognate tRNAs. As an additional level of quality control, many of the cytoplasmic ARSs have a double-sieve mechanism, through which they can edit mischarged tRNAs to prevent the misincorporation of amino acids into nascent proteins (Guo and Schimmel, 2012; Jakubowski, 2012). Mitochondrial ARSs, on the other hand, largely lack functional editing domains (with the exception of alanyl-tRNA synthetase (Gotz et al., 2011)), which has been suggested to indicate that mitochondrial protein synthesis is inherently less accurate than the cytoplasmic counterpart or that the organellar protein quality control is focused on another step (Roy et al., 2005).

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Amino acid analogs of suitable size and shape that can form key hydrogen bonds with ARSs have the propensity to be mischarged onto tRNAs and be inserted randomly into nascent proteins in place of the cognate amino acid. Such analogs have been widely used as experimental tools to study the effects of amino acid misincorporation and consequent protein misfolding (Schmollinger et al., 2013; Trotter et al., 2002). Accumulation of misfolded proteins can result in proteotoxic stress-induced cell death, which for example plays an important role in neurodegeneration (Dasuri et al., 2011).

Canavanine is one of the most used amino acid analogs with antiviral, antifungal, antibacterial and anticancer potential (Bence et al., 2003; Jang et al., 2002; Robertson et al., 1984; Rodgers and Shiozawa, 2008; Rosenthal, 1977; Rosenthal et al., 1989). It is a naturally occurring arginine analog, produced by leguminous plants as a defensive compound against predators. It can mistakenly incorporate into the nascent protein chains in place of arginine (Pines et al., 1981), but it contains an oxygen atom instead of methylene group, leading to irreversible disruption of protein confirmation. Such replacement of arginine residues with canavanine is well studied to cause cytoplasmic translation stress as a result of accumulation of misfolded proteins (Dasuri et al., 2011).

Early studies found that canavanine inhibited protein synthesis in rat liver and brain mitochondria (Winston and Bosmann, 1971) and in the mitochondria of *Saccharomyces cerevisiae* (Wilkie, 1970) but the molecular consequences of canavanine misincorporation in mitochondria have not been studied in detail. We present here an investigation of the effects of canavanine on mitochondrial protein synthesis and OXPHOS complex maintenance in human cells. We show that canavanine stalls mitoribosomes downstream of arginine codons and induces aberrant polypeptides. Furthermore, canavanine-induced protein misfolding destabilizes newly synthesized mitochondrial proteins, contributing to loss of OXPHOS complexes.

2. Materials and methods

2.1. Cell culture and treatments

The human neuroblastoma cell line, SH-SY5Y, was cultured in mixture of EMEM/F12 medium (1:1) supplemented with 10% fetal bovine serum, non-essential amino acids, penicillin/streptomycin, sodium pyruvate, D-glucose, uridine and GlutaMAX. The human osteosarcoma cells, 143B, were grown in DMEM medium with 10% fetal bovine serum, L-glutamine and penicillin/streptomycin. The primary human myoblasts were cultured in skeletal muscle myoblast cell growth medium (SkGM, Lonza). All experiments were performed in the presence of normal amount of arginine in the culture medium. 20 mM L-canavanine (CAN; Sigma) was used throughout this work, unless stated otherwise. To inhibit mitochondrial translation cells were treated with chloramphenicol (30 μg/ml) for 48 h.

2.2. SDS–PAGE and BN-PAGE

Protein extracts for SDS–PAGE analysis were prepared by lysing cells in RIPA buffer (1% NP40, 0.05% sodium deoxycholate and 0.1% sodium dodecyl sulfate in phosphate buffered saline) containing protease inhibitors. Lysates were incubated on ice for 20 min before centrifugation at 12,000 × *g* for 20 min at 4 °C. Protein samples were separated on 15% SDS–PAGE gels. Samples for BN-PAGE analysis were prepared as previously described (Schagger and von Jagow, 1991). All buffers were adjusted to pH 7.0 at 4 °C. Mitochondria were purified from digitonin treated cells. Samples were loaded on 6–15% Bis–Tris Native PAGE gels and electrophoresed. After SDS–PAGE or BN-PAGE, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane by semi-dry blotting using the Bio-Rad western transfer unit. The membranes were blocked in 5% milk in TBS–Tween 20 (0.1%). Proteins were immunoblotted with the indicated primary antibodies and corresponding secondary antibodies. Specific proteins were detected using antibodies against: Tom40 (Santa Cruz, sc-11414), Complex I subunit NDUFA9 (Mitosciences, MS111), Complex II subunit SDHA (Mitosciences, MS204), Complex III subunit UQCRC2 (Abcam, ab14745), Complex IV subunit 1 (Mitosciences, MS404), Complex IV subunit 2 (GeneTex, GTX62145), Complex IV subunit 3 (Abcam, ab138956), mtHSP60 (Santa Cruz, sc-1052), mtHSP70 (Abcam, ab53098), ClpP (LifeSpan BioSciences, LS-C138964), mtDnaJ (Abcam, ab181024) and GAPDH (Cell Signaling, 14C10). Enhanced chemiluminescent substrate and ChemiDoc imaging station (BioRAD) were used for signal detection. Quantification of the bands was performed by Image Lab Software (BioRAD).

2.3. Mitochondrial translation assay

In vivo or in-organello mitochondrial translation assays were performed as previously described (Fernandez-Silva et al., 2007; Leary and Sasarman, 2009). In brief, mitochondrial translation products were labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine (PRO-MIX, Amersham). For *in vivo* translation analysis, cells were preincubated in methionine- and cysteine-free medium for 30 min and then radiolabeled with 200 μCi/ml of PRO-MIX in the presence of 100 μg/ml anisomycine (Sigma) for 1 h. The cells were chased in the standard media for 10 min (pulse experiment) or for indicated time (chase experiment). To perform in-organelle translation assay, mitochondria were isolated and incubated with PRO-MIX at 37 °C for 1 h. In the experiments with amino acid analog, 20 mM L-canavanine was added 5 min before radiolabeling. Protein samples were analyzed on 12–20% gradient SDS–polyacrylamide gels. Radioactive signal was detected by storage phosphor screen using Typhoon and quantified with the ImageQuant v5.0 software (GE Healthcare). To confirm equal loading Coomassie staining was performed.

For nonradioactive measuring of mitochondrial translation rate, cultured SH-SY5Y neuroblastoma cells were incubated with 10 μg/ml of puromycin in presence of 100 μg/ml emetine. Puromycin incorporation into newly synthesized proteins was detected by immunoblotting using antibody to puromycin (Millipore, clone 12D10).

2.4. Mitoribosome profiling

Deep sequencing of mitoribosome-protected RNA fragments was performed as described previously (Rooijers et al., 2013). Briefly, SH-SY5Y neuroblastoma cells were pretreated with 20 mM canavanine for 1.5 h or with 1 M canavanine for 18 h, then cytoplasmic and mitochondrial translation were stopped by adding 100 mg/ml chloramphenicol and 200 mg/ml cyclohexamide. The lysed cells were treated with RNase I and fractionated on a sucrose gradient. Fractions enriched in monosomes were treated with proteinase K. The purified ribosome protected mRNA fragments (25–36 nucleotide length) were used for library preparation. Deep sequencing was performed on a HiSeq 2000 System (Illumina).

3. Results

3.1. Canavanine affects the stability of mitochondrial proteins and induces aberrant polypeptides

To investigate the effects of canavanine on mitochondrial protein synthesis in human cells, we labeled newly synthesized