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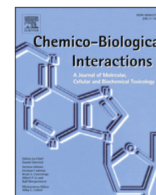

# Lon protease and eiF2α are involved in acute, but not prolonged, antiretroviral induced stress response in HepG2 cells

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Lon protease, an ATP dependent mitochondrial protease, is important in mitochondrial protein maintenance. Disruption of protein homeostasis and mitochondrial dysfunction is associated with lipodystrophy, metabolic syndrome and accelerated aging, and are commonly observed in patients on long term antiretroviral therapy. Sirtuin 3 (SIRT3) is a post-translational regulator of Lon and regulates antioxidant response. We previously showed the nucleoside analogues (NRTIs), Zidovudine (AZT; 7.1 μM), Stavudine (d4T; 4 μM), and Tenofovir (TFV; 1.2 μM) induced oxidative stress and mitochondrial dysfunction in human hepatoma (HepG2) cells at 24 h (h) and 120 h. We conducted a mitochondrial proteomic assessment of homeostasis in the same model, using the same NRTIs. Protein expression of Lon, SIRT3, heat shock protein (HSP) 60, phospho-eukaryotic translation initiation factor 2α (p-eIF2α; Ser51) and phospho-c-jun N-terminal kinase (p-JNK; Thr183/Tyr185) were quantified by western blots. The data showed all stress responses were significantly increased in HepG2 cells by all antiretroviral drugs at 24 h ($p < 0.0001$); however, at 120 h, a significant depletion in the ATP-dependent proteins Lon ($p = 0.00013$) and HSP60 ($p < 0.0001$) was observed. Proteins initiated by endoplasmic reticulum stress: p-eIF2α ($p = 0.001$) and p-JNK ($p = 0.0029$), were significantly reduced following prolonged treatment. SIRT3 was maintained at elevated levels in the treated cells following prolonged exposure ($p < 0.001$). We conclude that the ATP dependent proteins are more relevant to acute toxicity, while SIRT3 confers protection over prolonged periods of toxicity.

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## 1. Introduction

Mitochondrial integrity is imperative to the optimal functioning of a biological system. Functionality of mitochondria is maintained by tightly regulated stress and repair mechanisms. A progressive decline in the efficacy of these repair and stress responses is a characteristic of biological aging [8]. The result of impaired stress responses is reduced mitochondrial respiratory capacity and increased production of free radicals, leading to oxidative stress. The high oxidative environment created makes protein dense mitochondria susceptible to oxidative modification(s). Oxidatively modified proteins, if not cleared by proteolysis, begin to form occlusions and may result in aberrant structural changes to mitochondria [18]. The disruption to protein homeostasis in the mitochondria elicits cellular stress responses to recruit repair mechanisms and mitochondrial chaperones.

Cellular proteases and chaperones are essential for the removal of misfolded or aberrant proteins. Proteolytic removal of oxidized proteins from the mitochondrial matrix is largely achieved by the

*Abbreviations:* AAA+, ATPases associated with diverse cellular activities; AZT, Zidovudine; CHOP, CCAAT-enhancer-binding protein homologous protein; d4T, Stavudine; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; HSP, heat shock protein; JNK, c-jun N-terminal kinase; NRTI, nucleoside reverse transcriptase inhibitor; PERK, protein kinase RNA-like ER kinase; RBI, relative band intensity; SIRT3, Sirtuin 3; TFV, Tenofovir.

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evolutionarily conserved Lon protease (Lon). This mitochondrial protein, encoded for by the gene *LONP1*, belongs to the ATPases Associated with diverse cellular Activities (AAA+) protease family. Lon has the ability to recognise misfolded or damaged proteins by the exposed hydrophobic core of target proteins. The damaged protein is then proteolytically processed by Lon in an ATP-dependent manner [7]. Substrates of Lon are not exclusive to aberrant or misfolded proteins. The role of Lon in mitochondrial DNA turnover has been identified due to its selective degradation of mitochondrial transcription factor A [14,15]. The central role of Lon in mitochondrial protein maintenance indicates the potential of this protein in many diseases related to mitochondrial dysfunction [18].

Hypoxia, oxidative stress, endoplasmic reticulum (ER) stress and ATP availability are inducers of Lon expression. Lon is involved in acute oxidative stress response, however, its activity cannot be maintained during chronic stress [18]. There have been no correlations found between *LONP1* mRNA levels and Lon protein expression, suggesting posttranscriptional regulation of this protein [6]. This is common in multiple proteins involved in stress response as it provides a means to increase protein turnover without the time consuming synthesis, processing and exportation of *de novo* mRNA synthesis. The posttranslational regulation of Lon by Sirtuin 3 (SIRT3) was recently described by Ref. [6]. Silencing of SIRT3 in breast cancer cells led to an accumulation of Lon protein but not *LONP1* mRNA.

The sirtuin family of $NAD^+$ dependent deacetylases has been gaining attention for their role in longevity and metabolism. The mitochondrial localized SIRT3 is responsible for fat metabolism, antioxidant defence [9], mitochondrial integrity and function, and the mitochondrial unfolded protein response ($UPR^{mt}$) [19]. The function of SIRT3 in maintaining protein homeostasis is independent of the activity of the transcription factors CHOP and oestrogen receptor $\alpha$. This new mechanism of SIRT3 mediated $UPR^{mt}$ is believed to be mediated by SIRT3 antioxidant and mitophagy regulation [19]. A depletion of SIRT3 is associated with accelerated aging-associated disorders [16]. The overlapping role of SIRT3 and Lon in the maintenance of protein homeostasis is a scarcely described mechanism in acute and chronic stress response.

Studies have shown Lon interacts with other mitochondrial chaperones including heat shock protein (HSP) 60, and mitochondrial HSP70 [10,11]. Cellular chaperones such as HSPs confer resistance to stress conditions, promoting cell survival. The complex formed by Lon, HSP60 and mitochondrial HSP70 has been identified as a component in the transmission of ER stress to the mitochondria. Prolonged oxidative stress causes an increase in misfolded proteins which aggregate in the ER. The objective of the ER stress response is to inhibit or slow down protein synthesis, reducing the load of client proteins in the ER. Translational repression is achieved by phosphorylation of eukaryotic translation initiation factor 2$\alpha$ (eIF2$\alpha$). This protein is activated by protein kinase RNA-like ER kinase (PERK) or c-jun N-terminal kinase (JNK). Phosphorylation of eIF2$\alpha$ is elevated during oxidative stress and has been suggested as an ER response to mitochondrial stress [1].

The long term use of nucleoside reverse transcriptase inhibitors (NRTIs) in antiretroviral therapy has been associated with metabolic syndrome, lipodystrophy [4] and accelerated aging [22]. Mitochondrial dysfunction has been implicated as a molecular mechanism for these clinical outcomes [2]. Mitochondrial protein maintenance plays a critical role in retarding the development of NRTI-associated adverse health outcomes. Presently, only one study has quantified Lon expression in relation to antiretroviral use and lipodystrophy [20], however the response of SIRT3 and Lon in NRTI-induced hepatotoxicity has not been described. We previously showed that the NRTIs Zidovudine (AZT) and Tenofovir (TFV) induced acute oxidative stress in human hepatoma (HepG2) cells, while Stavudine (d4T) and TFV elevated oxidative stress markers following prolonged exposure [17]. This was accompanied by a heightened antioxidant response. We replicated this model to assess the role of Lon and SIRT3 in the mitochondrial protein homeostatic response in HepG2 cells following acute [24 h (h)] and prolonged (120 h) exposure to NRTIs: AZT, d4T and TFV.

## 2. Methods

### 2.1. Cell culture and treatment

Human hepatoma (HepG2) cells were cultured in 25 $cm^3$ cell culture flasks in Eagles minimum essential medium (Lonza; BioWhittaker, Johannesburg, South Africa) supplemented with 10% foetal calf serum, 1% L-glutamine and 1% penstrepfungizone. Cells were allowed to reach 80% confluence prior to treatment. Stocks of antiretroviral drugs were made in DMSO (10 mM). Cells were treated with maximum plasma level concentrations [23,24] of AZT (7.1 μM); d4T (4 μM); and TFV (1.2 μM). Antiretroviral drugs used were obtained from the NIH AIDS reagents program. Cells were exposed to NRTIs for 24 h (h) and 120 h prior to harvesting for protein extraction.

### 2.2. Protein preparation

The protein expression of key proteins involved in mitochondrial protein homeostasis was assessed by the western blot technique. Treated cells were rinsed twice with 0.1 M phosphate saline buffer (PBS) and incubated in 200 μl Cytobuster reagent (Novagen, San Diego, CA) for 10 min (min) on ice. The cell lysates were transferred to 1.5 ml micro-centrifuge tubes and centrifuged (13,000× g; 10 min). Crude protein extracts were aspirated and quantified using the bicinchoninic acid assay and standardized to 1.2 mg/ml. Proteins samples were then boiled in Laemelli buffer [$dH_2O$, 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, b-mercaptoethanol, 1% bromophenol blue] for 5 min.

### 2.3. SDS-PAGE and western blot

The prepared protein samples were separated on SDS polyacrylamide gels (4% stacking, 7.5% resolving) by electrophoresis (150 V; 1 h). Separated proteins were transferred to nitrocellulose membranes using the TransBlot Turbo Transfer system (Bio-Rad, Hercules, CA, USA) at 400 mA for 45 min. Following transfer, nitrocellulose membranes were incubated in blocking solution (5% non-fat dry milk; 1 h; RT) on a shaker. Membranes were then incubated in primary antibody (1:1000 dilution in 5% non-fat dry milk) for 1 h at RT on a shaker, followed by overnight incubation at 4 °C. Antibodies were obtained from Cell Signalling Technology (Beverly, MA, USA) and Sigma Aldrich (St Louis, MO, USA): anti-LONP1 (HPA002034), anti-HSP60 (D307), anti-SIRT3 (C73E3), anti-phospho-eiF2$\alpha$ (Ser51; #3597), anti-phospho-JNK (Thr183/Tyr185; #9251). Following probing with primary antibody, membranes were rinsed (5 × 10 min) with Tween 20-Tris buffered saline (TTBS). The membranes were then incubated in horse radish peroxidase-conjugated secondary antibody (1: 10,000 dilution in 5% non-fat dry milk) for 1 h, at RT on a shaker. The membranes were rinsed again (5 × 10 min) in TTBS prior to chemiluminescent detection.

Protein bands were visualized using Clarity Western ECL detection reagent (Bio-Rad, Hercules, CA) and images were captured on the Alliance 2.7 gel documentation system (UviTech, Cambridge, UK).

Densitometric analysis was done using the UviBand Analysis

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