Biochemical and Biophysical Research Communications xxx (2015) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Long-lived species have improved proteostasis compared to phylogenetically-related shorter-lived species

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ARTICLE INFO

*Article history:*
Received 9 January 2015
Available online xxx

*Keywords:*
Protein homeostasis
Autophagy
Proteasome
Heat shock response
Long-lived species

ABSTRACT

Our previous studies have shown that the liver from Naked Mole Rats (NMRs), a long-lived rodent, has increased proteasome activity and lower levels of protein ubiquitination compared to mice. This suggests that protein quality control might play a role in assuring species longevity. To determine whether enhanced proteostasis is a common mechanism in the evolution of other long-lived species, here we evaluated the major players in protein quality control including autophagy, proteasome activity, and heat shock proteins (HSPs), using skin fibroblasts from three phylogenetically-distinct pairs of short- and long-lived mammals: rodents, marsupials, and bats. Our results indicate that in all cases, macroautophagy was significantly enhanced in the longer-lived species, both at basal level and after induction by serum starvation. Similarly, basal levels of most HSPs were elevated in all the longer-lived species. Proteasome activity was found to be increased in the long-lived rodent and marsupial but not in bats. These observations suggest that long-lived species may have superior mechanisms to ensure protein quality, and support the idea that protein homeostasis might play an important role in promoting longevity.



## 1. Introduction

Disruptions in proteostasis, a set of cellular mechanisms that maintain the stability of the proteome [1], can result in an increased burden of misfolded proteins, leading to toxic oligomers and the accumulation of insoluble protein aggregates, thought to play a role in many chronic diseases, including age-related neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease, among others [2].

The main players in proteostasis include the ubiquitin/proteasome system, autophagy, and heat shock chaperones. The ubiquitin/proteasome pathway is involved in the removal of short-lived proteins that have been damaged and/or misfolded, while autophagy is crucial for the degradation and recycling of long-lived proteins, macromolecular aggregates, and damaged intracellular organelles. Protein chaperones, in turn, promote protein quality control by covering hydrophobic regions of proteins that are exposed during the normal and dynamic process of unfolding/refolding of proteins, thus assuring that proteins acquire a stable folded conformational state and do not oligomerize and aggregate. These mechanisms are known to decline with age, and this might be at least partially responsible for the increased accumulation of oxidatively and otherwise damaged proteins and aggregates with

*Abbreviations:* NMR, naked mole rat; LB, little brown bat; EB, evening bat; SG, sugar glider; Opo, laboratory opossum.

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http://dx.doi.org/10.1016/j.bbrc.2015.01.046
0006-291X/

advancing age [3]. Dietary restriction (DR), the best characterized manipulation that extends lifespan and healthspan in mice, has been shown to increase the heat shock response and autophagy, processes that would be predicted to reduce protein misfolding and the accumulation of protein oligomers/aggregates.

Using a comparative biology approach, previous studies including our own, have shown that proteins present in extracts from the liver of a long-lived rodent and bats have better resistance to urea-induced unfolding when compared to mice, a shorter-lived species with similar body weight [4,5]. While our previous work was done *in vitro* with liver extracts, in this report we used cultured skin fibroblasts to further evaluate whether an enhancement of several proteostatic mechanisms (macroautophagy, proteasome activity, and heat shock chaperones) is associated with longevity. Therefore, in this work we are comparing 3 pairs of long- and short-lived species: rodents [naked mole rats (NMRs; maximum lifespan (mls) ~30 year) vs. mice (mls ~4y), marsupials (sugar glider, mls ~18y vs. laboratory opossum (mls ~5y), and bats (evening bat, mls ~6y vs. little brown bat, mls ~34y).

## 2. Materials and methods

### 2.1. Species

The species studied were chosen based on their 1) well established longevity, 2) similar body body size, and 3) representation of a broad phylogenetic coverage within mammals, to ensure generality of our conclusions. The 3 clades chosen are: **Rodents**: laboratory mice [*Mus musculus*, 35gr and 4y], vs. Naked mole rats (NMRs) [*Heterocephalus glaber,* 30 gr and ~30y]; **Bats**: evening bat (EB) [*Nycticeius humeralis,*11gr and 6y] vs. little brown bat (LBB) [*Myotis lucifugus,* 8gr and 34y]; and **Marsupials:** laboratory opossum (opo) [*Monodelphis domestica,* 150 gr and 4,75y] vs. sugar glider (SG) [*Petaurus brevicaudus,* 100gr and 18y] (Supplemental Fig. 1).

### 2.2. Cell culture

Skin fibroblasts from long- and short-lived species were obtained from the Comparative Biology of Aging Core in the San Antonio Nathan Shock Center. Briefly, cells were prepared by enzymatic digestion of skin from young animals, and cultured in low-glucose Dulbecco's Modified Eagle's Medium (DMEM) and 10% Cosmic Calf Serum (Hyclone Laboratories, Logan, UT, USA) in a 37 °C incubator with a gas phase of 21% $O_2$, 5% $CO_2$, with the exception of NMR and mouse fibroblasts, which were cultured at 35 °C [6]. In all experiments, cells were used between passage 4 and 8. For NMR fibroblast cultures, we used Biocoat collagen I-coated tissue culture dishes (Advance Biometrix, San Diego, CA).

### 2.3. Protein degradation flux and macroautophagy

Degradation of long lived proteins was measured by radioisotopic pulse-chase labeling as described by Massey et al., 2008 [7]. Briefly, cells were incubated for 48 h in DMEM containing 2 μCi/ml $^3$H-valine (Perkin–Elmer, MA, USA). Two wells were harvested to determine total radioactivity incorporated into protein at the zero time point and the remaining wells received the chase medium: DMEM containing 2.8 mM cold valine in the presence or absence of serum (serum free), 10 mM 3-methyladenine (3-MA) for inhibition of macroautophagy and 20 mM/100 μM ammonium chloride/leupeptin (AC/L) for lysosomal inhibition. Cells were then chased for 4, 8, 12, or 24 h. Proteolysis was measured as the amount of acid precipitable radioactivity transformed into acid soluble radioactivity during the chase period. Macroautophagy was calculated as the percentage of lysosome-mediated protein degradation sensitive to 3-MA inhibition.

### 2.4. Western blot analysis

Briefly, total cell lysates were prepared in RIPA buffer supplemented with protease and protein phosphatase inhibitors (Calbiochem, La Jolla, CA) and subjected to SDS-PAGE followed by transferring to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were incubated with antibodies specific for: S6, phospho S6 (P-S6), LC3, heat shock chaperones: 90, 70, 40, and 27 (Cell Signaling Technology, Inc Danvers, MA), Actin (MP Biomedicals, Solon, OH), 20S proteasome subunit [8]. The intensity of the bands was quantified by densitometry using Imagelab software (Bio-rad, Hercules, CA).

### 2.5. Heat shock response

A heat shock response was induced in fibroblasts by incubation at 41 °C for 1hr in 5% $CO_2$/95% air [9]. Then cells were quickly transferred to a 35 °C (mouse and NMR fibroblasts) or 37 °C (bats and marsupials) incubator (5% $CO_2$/95% air) and allowed experimental groups to recover for 2, 4, 6,or 24 h. The control group (0 time) was not exposed to heat shock. Fibroblasts were harvested using RIPA buffer supplemented with protease inhibitors (Calbiochem, Billerica, MA) and 30 μg of protein were subjected to western blot analysis. Hsp90, Hsp70, Hsp40, and Hsp27 were measured by Western blot analysis using specific antibodies for each of these proteins. The level of each protein was calculated by quantification of each band relative to the loading control actin, with attention to quantification of images where signals were not saturated.

### 2.6. 20S proteasome activity assay

Fibroblasts were homogenized in homogenization buffer (50 mM Tris-CL, pH 8.0; 1 mM EDTA; 0.5 mM DTT) and protein concentrations were measured by BCA assay. For each sample, 100 μg total protein was assayed in triplicate in 96-well plates using a 20S proteasome fluorometric (AMC) assay kit as per instructions from the vendor (Calbiochem, Billerica, MA). In brief, the release of free AMC from the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC was measured over time at 37 °C using a microplate fluorescence spectrophotometer. 20S activity was calculated by the slope of free AMC release over time after ~10 min period of normalization. 20S proteasome specific activity was calculated by normalizing 20S activity to the quantity of 20S proteasome as measured by Western blot; data were expressed as AMC release per second per mg of protein. A proteasome inhibitor, Lactacystin, was used to verify proteasome-driven proteolysis.

## 3. Results

### 3.1. Enhanced macroautophagy in fibroblasts from long-lived species

Because autophagy plays a central role in proteostasis [10], we measured autophagy under both basal conditions and induced by serum deprivation, by monitoring the degradation of radioactively-labeled long-lived proteins (autophagy flux; Fig. 1A), as described by Massey et al., 2008. Macroautophagy was measured in the presence of 3-methyladenine (3-MA) as described by Wang et al. (2008) [11]. Dose response curves reveal similar species sensitivity to this inhibitor (data not shown).

Our analysis showed that macroautophagy is enhanced in fibroblasts from all long-lived species. Specifically, fibroblasts from



Please cite this article in press as: H. Pride, et al., Long-lived species have improved proteostasis compared to phylogenetically-related shorter-lived species, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.01.046