Biochemical and Biophysical Research Communications 434 (2013) 815–819

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Elevated protein carbonylation and oxidative stress do not affect protein structure and function in the long-living naked-mole rat: A proteomic approach

Eric M. De Waal [a], Hanyu Liang [b], Anson Pierce [c], Ryan T. Hamilton [e,h], Rochelle Buffenstein [d,e], Asish R. Chaudhuri [d,f,g,*]

[a] Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA 19104, USA
[b] Department of Medicine-Diabetes Division, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA
[c] Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX 77555, USA
[d] Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA
[e] Barshop Institute of Longevity and Aging Studies, South Texas Veterans Health Care System, San Antonio, TX 78229, USA
[f] Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA
[g] Geriatric Research, Education, and Clinical Center, South Texas Veterans Health Care System, San Antonio, TX 78229, USA
[h] Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA

ARTICLE INFO

Article history:
Received 7 April 2013
Available online 22 April 2013

Keywords:
Naked-mole rat
Peroxiredoxin 1
Triosephosphate isomerase
Protein carbonylation
Oxidative stress

ABSTRACT

The 'oxidative stress theory of aging' predicts that aging is primarily regulated by progressive accumulation of oxidized macromolecules that cause deleterious effects to cellular homeostasis and induces a decline in physiological function. However, our reports on the detection of higher level of oxidized protein carbonyls in the soluble cellular fractions of long-living rodent naked-mole rats (NMRs, lifespan ~30 yrs) compared to short-lived mice (lifespan ~3.5 yrs) apparently contradicts a key tenet of the oxidative theory. As oxidation often inactivates enzyme function and induces higher-order soluble oligomers, we performed a comprehensive study to measure global protein carbonyl level in different tissues of age-matched NMRs and mice to determine if the traditional concept of oxidation mediated impairment of function and induction of higher-order structures of proteins are upheld in the NMRs. We made three intriguing observations with NMRs proteins: (1) protein carbonyl is significantly elevated across different tissues despite of its exceptional longevity, (2) enzyme function is restored despite of experiencing higher level of protein carbonylation, and (3) enzymes show lesser sensitivity to form higher-order non-reducible oligomers compared to short-living mouse proteins in response to oxidative stress. These observations were made based on the global analysis of protein carbonyl and identification of two heavily carbonylated proteins in the kidney, triosephosphate isomerase (TPI) and cytosolic peroxiredoxin (Prdx1). These un-expected intriguing observations thus strongly suggest that oxidative modification may not be the only criteria for impairment of protein and enzyme function; cellular environment is likely be the critical determining factor in this process and may be the underlying mechanism for exceptional longevity of NMR.

1. Introduction

Proteins in general are potential targets for oxidative insult as most of the amino acids in proteins are sensitive to oxidation. Since good quality of proteins plays an important role in maintenance of normal cellular homeostasis, it is conceivable that inactivation of function by oxidative modification is likely to cause deleterious effects on cellular function during aging. Previous studies in short-lived mice have shown that aging is associated with increase in oxidative damage to proteins as well as a concomitant misfolding and functional impairment of the proteins/enzymes [1–4]. However, we also demonstrated in parallel that the exceptionally long-lived mouse-sized naked mole-rats (NMRs; *Heterocephalus glaber*) [5] exhibit higher levels of protein carbonylation to cytosolic proteins even at a young age, compared to young short-lived mice [6,7]. Yet, in sharp contrast to the age-related accumulation of oxidative damage in short-lived mice, NMRs do not demonstrate an accumulation of oxidized proteins and lipids throughout their lifespan even though they maintain high-steady state levels from

Abbreviations: NMRs, naked-mole rats; TPI, triosephosphate isomerase; Prdx1, Peroxiredoxin 1; FTC, fluorescein-5-thiosemicarbazide.

* Corresponding author. Address: Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center at San Antonio, Texas Research Park Campus, 15355 Lambda Drive, San Antonio, TX 78245-3207, USA. Fax: +1 210 562 6110.
E-mail address: chaudhuria@uthscsa.edu (A.R. Chaudhuri).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.04.019

a young age [2,6,8]. Moreover, young and old NMR proteins showed structural resistance to misfolding as compared to short lived mice proteins when were treated with urea, a chaotropic agent that disrupts intra-molecular interactions mediated by non-covalent forces, such as hydrophobic bonds, Van Der Waals forces and electrostatic stabilization [2]. Taken together, all of these data thus suggest that NMRs have the ability to maintain their protein structure during challenge with cellular and chemical stressors.

Oxidative stress and protein modifications have been considered as one of the critical components in inactivation of proteins/enzyme function during mouse aging [9]. Our earlier report related to elevated level of protein carbonylation observed in liver of NMRs led us to determine whether protein carbonylation has any negative impacts on structure and function of protein and enzyme. In this study, we report three intriguing observations; first, protein carbonylation is significantly elevated across different tissues despite the fact that NMRs have exceptional longevity, secondly, TPI enzyme (isolated from NMRs kidney) which had experienced massive oxidative insult (e.g., protein carbonylation) did not lose its enzymatic function, instead, its enzyme function was activated, and third, both TPI and Prdx1 showed lesser sensitivity to form higher-order non-reducible oligomers when were challenged with *in vitro* oxidative stress. These intriguing observations thus strongly suggest two important biological phenomenon; first, perturbation of structure and function of protein and enzyme may not always be dependent on oxidative modifications; instead it may depend on whether the modification has influence on the catalytic, structural integrity or regulatory sites of the protein or enzyme, and second, the cellular environment might have critical role in protecting proteins and enzymes from oxidative-stress-dependent inactivation of function as well as preventing accumulation of aberrant non-reducible protein oligomers. All of these plausible mechanism(s) might be used by long-living NMRs to protect proteins from oxidative insult.

2. Materials and methods

2.1. Animals

Wild-type C57BL/6 mice were fed *ad libitum* a standard NIH-31 chow and maintained in microisolator cages on a 12-h dark/light cycle. NMRs were from the well-characterized colonies maintained by Dr. Buffenstein's laboratory at the University of Texas Health Science Center at San Antonio. Comparisons were made between young, physiologically age-matched C57BL/6 mice (~0.3 year) and naked mole-rats (~2 years). Animals were killed by anesthesia; liver, heart, and kidney tissues were excised and snap frozen in liquid nitrogen and stored at −80 °C until use. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and the subcommittee for animal studies at Audie L. Murphy Memorial Veterans Hospital (San Antonio, TX).

2.2. Protein carbonylation

Protein carbonyl levels from cytosolic fraction of liver, heart, and kidney from both mice and NMRs were determined using gel electrophoresis as previously described [10] and with slight modification [3]. Snap frozen tissues isolated from mice/NMRs were placed in ice-cold 20 mm phosphate buffer, pH 6.0. Tissues were then homogenized by sonication in deaerated buffer [20 mm sodium phosphate buffer pH 6.0 containing 0.5 mm $MgCl_2$, 1 mm EDTA and protease cocktail inhibitors (500 μm AEBSF, HCl, 150 nm aprotinin, 0.5 mm EDTA, disodium salt and 1 μm leupeptin hemisulfate)] and centrifuged at 4 °C for 1 h at 100, 000*g*. The supernatant (cytosolic fraction) was treated with 1% streptomycin sulfate and incubated at 37 °C for 10 min. The solution was centrifuged at 11, 000*g* for 10 min at room temperature to remove nucleic acids, which contain reactive carbonyl groups. The cytosolic extracts were diluted to 1 mg $*$ mL^{-1}, and were mixed with 0.3 m guanidine followed by incubating with fluorescein-5-thiosemicarbazide (FTC; 1 mm) at 37 °C for 150 min in the dark. The proteins were precipitated with an equal volume of 20% chilled trichloroacetic acid (v:v) and centrifuged at 16 000*g* for 5 min at 25 °C. The pellets were then resuspended and washed five times with 100% ethanol/ethyl acetate (1:1) (v:v) to remove the unbound free FTC. The final pellets were then dissolved in phosphate buffer pH 8.0 containing 0.5 mm $MgCl_2$, 1 mm EDTA and 8 m urea. The concentration of the protein was determined by the Bradford assay and 15–25 μg of protein was subjected to 12% SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). After electrophoresis, the image of the fluorescent protein on the gel was captured with the Typhoon 9400 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using an excitation wavelength of 488 nm and an emission filter at 520 nm with a 40 nm bandpass. The intensity of fluorescence for each lane (from top to bottom of the lane) was calculated using ImageQuant 5.0 (Molecular Dynamics, Sunnyvale, CA, USA) software. The same area was used for all samples, so any change in volume is the result of FTC incorporation. The final fluorescence intensity values are expressed in milligram protein, by adjusting the original amount (15–25 μg) loaded onto the SDS–PAGE gel.

2.2.1. Mass spectrometric analysis

The identification of proteins was determined by HPLC–ESI–MS/MS after separation of the FTC-labeled cytosolic NMR kidney cytosolic proteins (150 μg) by 2D-gel electrophoresis using the method as described earlier [3]. In brief, FTC-labeled protein spots from NMR kidney cytosolic proteins were excised from the 2D gel, digested with modified trypsin (Promega) and analyzed using an Agilent 1100 series HPLC-PE Sciex QSTAR^{TM} hybrid LC-MS/MS Quadrupole TOF mass spectrometer. MS/MS data was analyzed using the MASCOT database search engine.

2.2.2. Triosephosphate isomerase activity

TPI activity was measured by a spectrophotometric based assay by monitoring the conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate by coupling the product to excess α-glycerophosphate dehydrogenase [11]. One unit of TPI activity is defined as the amount required for the conversion of 1 μmol of glyceraldehyde-3-phosphate into dihydroxyacetone phosphate per minute at 30 °C.

2.2.3. Determination of shift of monomer to oligomeric state of triosphosphate isomerase and peroxiredoxin1 in response to oxidative stress

Kidney cytosolic fractions from mouse and naked-mole rat were treated with or without of 0.03 mM Fe^{+2}/7.5 mM ascorbate for 1 h in the dark at 37 °C followed by loading equal amounts of proteins onto SDS-gel for electrophoresis. After the gel electrophoresis, proteins were transferred to PVDF membrane and Western blot was performed using antibodies against TPI and Prdx1 to determine the status of the shift of monomeric to oligomeric state in response to oxidative stress.

2.3. Statistical analysis

Results are presented as means ± SEM. Data were analyzed by student's *t-test* or by one-way ANOVA with Newman Keul's

multiple comparison test. Statistical significance for all analysis was set at $p < 0.05$.

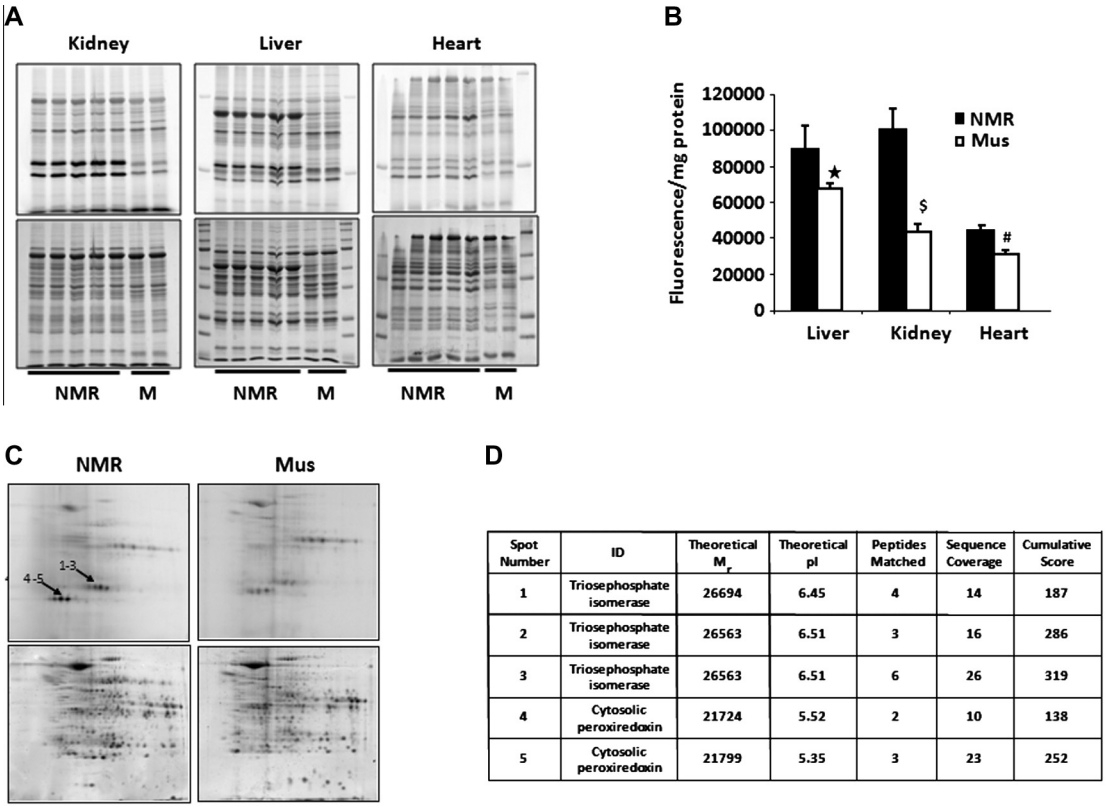
3. Results and discussion

In this study, we measured global protein carbonylation in the cytosolic fraction of liver, kidney and heart tissues of short-lived mice and long-lived NMRs by a fluorescence-based approach [3,10]. As shown in Fig. 1A and B, protein carbonyls were elevated in all three NMR tissues as compared to the mouse cytosolic proteins. Interestingly, the global elevation in protein carbonyl level observed in kidney was mainly contributed by two distinct molecular weights proteins with different isoelectric points (Fig. 1C) as determined by 2D gel electrophoresis.

Next, we were interested to identify those target proteins that were heavily carbonylated in kidney (Fig. 1C). The five spots that showed increase in protein carbonylation were analyzed by capillary HPLC-electrospray ionization tandem mass spectrometry (HPLC–ESI–MS/MS) on a Thermo Fisher LTQ or LTQ-Orbitrap Velos H/ETD and were found to be triosephosphate isomerase (TPI) and cytosolic peroxiredoxin (Prdx1) (Fig. 1D).

Since TPI is an important glycolytic enzyme that converts dihydroxyacetone phosphate to glyceraldehyde 3-phosphate and is known to undergo carbonylation during aging [12], we measured the functional activity of this enzyme in the presence of α-glycerophosphate dehydrogenase [11]. Interestingly, we observed 30% higher activity of TPI in kidneys of NMRs compared to mice (Fig. 2A). This un-expected observation suggests that the amino acids (e.g., cysteine or lysine) present in the active site of the TPI are either protected from carbonylation or the oxidation might occur at a distinct region (other than the active site) which has positive influence over the catalytic site to facilitate enzyme-substrate reaction. There is another strong possibility that NMRs might have enriched cellular environment which prevents inactivation of function of TPI. In order to address this biological phenomenon, we challenged the equal amount of cytosolic protein fractions (100,000*g* supernatant) of mouse and NMRs with Fe^{+2}/ascorbate to determine if the hydroxyl radical generation from metal-catalyzed oxidation reaction has any differential effects on TPI of mouse and NMRs in terms of formation of higher-order oligomeric structures. Based on Fig. 2B, there is clear indication that that is the case as metal catalyzed oxidation reaction indeed has differential effects on TPI. TPI of mouse showed more sensitivity in forming non-reducible higher-order oligomeric state than NMRs in response to oxidative stress; approximately 40% elevation of non-reducible soluble oligomers of TPI was detected in mouse in response to oxidative stress while no increase was observed in case of NMRs. These two sets of data (Fig. 2A and B) collectively suggest that cellular environment might have major role in maintaining functional structure of protein and enzyme.

Similarly, we also studied the oligomeric state of another enzyme, Prdx1 which was found carbonylated in kidney of NMRs (Fig. 1). Prdx in general, reduces hydrogen peroxide [13–15] through thiol-disulfide exchange. Prdx converts hydrogen peroxide to water through the oxidation of the peroxidatic cysteine residue to sulfenic acid and the concomitant condensation of the resolving cysteine residue to form a disulfide which is then coupled to thioredoxin enzyme or vitamin C for its reduction



Spot Number	ID	Theoretical M_r	Theoretical pI	Peptides Matched	Sequence Coverage	Cumulative Score
1	Triosephosphate isomerase	26694	6.45	4	14	187
2	Triosephosphate isomerase	26563	6.51	3	16	286
3	Triosephosphate isomerase	26563	6.51	6	26	319
4	Cytosolic peroxiredoxin	21724	5.52	2	10	138
5	Cytosolic peroxiredoxin	21799	5.35	3	23	252

Fig. 1. Protein carbonylation is elevated in different tissues from naked-mole rats (A) Protein carbonylation (15 μg protein/lane) was measured in cytosolic fractions isolated from liver, kidney, and heart tissues from young mice (M, 4–5 mo) and naked-mole rats (NMR, 2 yrs) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using the fluorescent probe, fluorescein-5-thiosemicarbazide (FTC). The figures in the top panels represent SDS–PAGE of the carbonylated proteins in different tissues and the lower panels represent coomassie blue staining. (B) Quantification of the carbonylated proteins from *Mus* (solid bars) and NMRs (open bars) are shown. Data are represented as mean ± SD of 4 samples/group. Data was analyzed by student's *t*-test ($^{*}p < 0.05$; $^{\#}p < 0.01$ and $^{\$}p < 0.001$). (C) Two-dimensional gel electrophoresis of FTC-labeled kidney cytosolic fraction from M and NMRs (the spots are indicated by arrows). (D) Identification of the carbonylated proteins in NMRs kidney cytosolic fraction by high-performance liquid chromatography and electrospray mass spectroscopy.

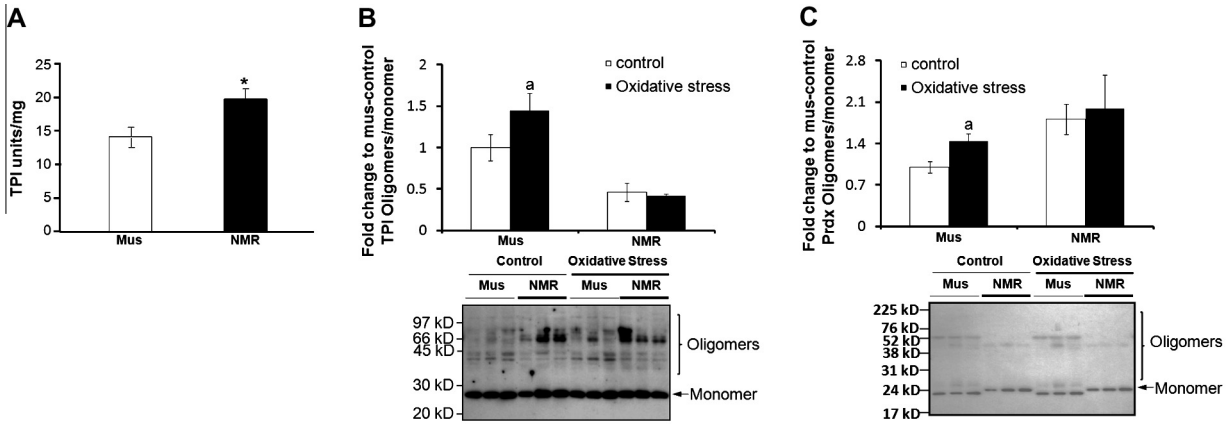


Fig. 2. Protein structure and function are maintained in kidneys from naked-mole rats. (A) Triosephosphate isomerase activity was measured in kidney cytosolic fractions from mice (M) and naked-mole rats (NMRs) by a spectrophotometric based assay. Results are expressed in mean ± SD ($n = 4$) and analyzed by student's t-test ($^*p < 0.01$). Kidney cytosolic fractions treated with or without 0.03 mM Fe^{+2}/7.5 mM ascorbate (oxidative stress) were run under reducing conditions on SDS–PAGE blotted against. (B) TPI and (C) Prdx2 ($n = 3$). (C) Quantification of data and comparison of data was performed as a ratio of the oligomers/monomer for both (B) TPI and (C) Prdx2 based on western blots of oxidized versus non oxidized Mouse and NMR cytosolic extracts. Results are mean ± SEM of 3 samples/group with significant differences obtained by a student t-test ($^*p < 0.05$).

[14–16]. We wanted to determine whether the cellular environment of NMRs uses its protective machinery to prevent conversion of Prdx1 from monomeric to oligomeric state. We therefore challenged the kidney cytosolic proteins of both mice and NMRs with iron/ascorbate followed by Western blotting as described in Fig. 2B with an antibody against Prdx1. The iron/ascorbate system generates hydroxyl radicals that can initiate oligomeric state of proteins via non-reducible oxidative modifications. We found that the conversion of monomeric to oligomeric state for Prdx1 was increased when the mouse proteins were challenged with Fe^{+2}/ascorbate. However, no changes for Prdx1 of NMRs were detected under challenged condition. In addition to these, we also observed slower mobility of Prdx1 in NMRs (Fig. 2C) suggesting that NMR Prdx1 might undergo differential post translational modifications which can increase its molecular mass (e.g., glycosylation) [17]. All of these data (Fig. 2B and C) thus strongly suggest that NMRs have a protective cellular environment which restores enzyme function and prevents formation of oligomers during oxidative stress.

In conclusion, we report an unconventional observation from a comparative study between NMRs and laboratory C57Bl/6 mice that protein oxidation might not induce deleterious effects on structure and function of proteins and enzymes. The cellular environment of NMRs is likely be the critical factor in modulating structure and function of protein and enzyme. The activation of NRF2 pathway (which increases the transcription of the antioxidant response element genes, proteasome, antioxidants and autophagy) and efficient maintenance of protein homeostasis could be potential plausible mechanisms for these processes as both of these were found activated and efficiently maintained in NMRs [18,19]. These could be potential mechanisms in explaining exceptional longevity of NMRs and their extended health well into the third decade of life [20]. Further investigation into the mechanisms particularly the role of the chaperones and redox machinery related to maintaining functional structure and protecting proteins from misfolding and aggregation may provide important insight about the slow aging process in NMRs.

Acknowledgments

This work was supported by the National Institutes of Health/National Institute on Aging pilot Grants K07 AG025063 04 (to A.C.) and from an NIH/NIA RO1AG-022891 (to R.B.). The following funding sources had no involvement in the interpretation, representation or publication of the work other providing the funding necessary.

References

[1] B. Chakravarti, D.N. Chakravarti, Oxidative modification of proteins: age-related changes, Gerontology 53 (2007) 128–139.
[2] V.I. Perez, R. Buffenstein, V. Masamsetti, S. Leonard, A.B. Salmon, J. Mele, B. Andziak, T. Yang, Y. Edrey, B. Friguet, W. Ward, A. Richardson, A. Chaudhuri, Protein stability and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole-rat, Proc. Natl. Acad. Sci. USA 106 (2009) 3059–3064.
[3] A.R. Chaudhuri, E.M. de Waal, A. Pierce, H. Van Remmen, W.F. Ward, A. Richardson, Detection of protein carbonyls in aging liver tissue: a fluorescence-based proteomic approach, Mech. Ageing Dev. 127 (2006) 849–861.
[4] K. Dasuri, P. Ebenezer, L. Zhang, S.O. Fernandez-Kim, A.J. Bruce-Keller, W.R. Markesbery, J.N. Keller, Increased protein hydrophobicity in response to aging and Alzheimer disease, Free Radic. Biol. Med 48 (2010) 1330–1337.
[5] R. Buffenstein, The naked mole-rat: a new long-living model for human aging research, J. Gerontol. A Biol. Sci. Med. Sci. 60 (2005) 1369–1377.
[6] A. Bhattacharya, S. Leonard, S. Tardif, R. Buffenstein, K.E. Fischer, A. Richardson, S.N. Austad, A.R. Chaudhuri, Attenuation of liver insoluble protein carbonyls: indicator of a longevity determinant?, Aging Cell 10 (2011) 720–723
[7] B. Andziak, T.P. O'Connor, W. Qi, E.M. DeWaal, A. Pierce, A.R. Chaudhuri, H. Van Remmen, R. Buffenstein, High oxidative damage levels in the longest-living rodent, the naked mole-rat, Aging Cell 5 (2006) 463–471.
[8] B. Andziak, R. Buffenstein, Disparate patterns of age-related changes in lipid peroxidation in long-lived naked mole-rats and shorter-lived mice, Aging Cell 5 (2006) 525–532.
[9] V.I. Perez, A. Pierce, E.M. de Waal, W.F. Ward, A. Bokov, A. Chaudhuri, A. Richardson, Detection and quantification of protein disulfides in biological tissues a fluorescence-based proteomic approach, Methods Enzymol. 473 (2010) 161–177.
[10] B. Ahn, S.G. Rhee, E.R. Stadtman, Use of fluorescein hydrazide and fluorescein thiosemicarbazide reagents for the fluorometric determination of protein carbonyl groups and for the detection of oxidized protein on polyacrylamide gels, Anal. Biochem. 161 (1987) 245–257.
[11] E.E. Rozacky, T.H. Sawyer, R.A. Barton, R.W. Gracy, Studies on human triosephosphate isomerase. I. Isolation and properties of the enzyme from erythrocytes, Arch. Biochem. Biophys. 146 (1971) 312–320.
[12] P.M. Yuan, J.M. Talent, R.W. Gracy, Molecular basis for the accumulation of acidic isozymes of triosephosphate isomerase on aging, Mech. Ageing Dev. 17 (1981) 151–162.
[13] S.W. Kang, S.G. Rhee, T.S. Chang, W. Jeong, M.H. Choi, 2-Cys peroxiredoxin function in intracellular signal transduction: therapeutic implications, Trends Mol. Med. 11 (2005) 571–578.

[14] S. Hirotsu, Y. Abe, K. Okada, N. Nagahara, H. Hori, T. Nishino, T. Hakoshima, Crystal structure of a multifunctional 2-Cys peroxiredoxin heme-binding protein 23 kDa/proliferation-associated gene product, Proc. Natl. Acad. Sci. USA 96 (1999) 12333–12338.
[15] E. Schroder, J.A. Littlechild, A.A. Lebedev, N. Errington, A.A. Vagin, M.N. Isupov, Crystal structure of decameric 2-Cys peroxiredoxin from human erythrocytes at 1.7 Å resolution, Structure 8 (2000) 605–615.
[16] A. Hall, P.A. Karplus, L.B. Poole, Typical 2-Cys peroxiredoxins–structures, mechanisms and functions, FEBS J. 276 (2009) 2469–2477.
[17] S. Lee, Y. Shin, J. Marler, M.C. Levin, Post-translational glycosylation of target proteins implicate molecular mimicry in the pathogenesis of HTLV-1 associated neurological disease, J. Neuroimmunol. 204 (2008) 140–148.
[18] K.N. Lewis, J. Mele, J.D. Hayes, R. Buffenstein, Nrf2, a guardian of healthspan and gatekeeper of species longevity, Integr. Comp. Biol. 50 (2010) 829–843.
[19] K.A. Rodriguez, Y.H. Edrey, P. Osmulski, M. Gaczynska, R. Buffenstein, Altered composition of liver proteasome assemblies contributes to enhanced proteasome activity in the exceptionally long-lived naked mole-rat, PLoS One 7 (2012) e35890.
[20] R. Buffenstein, Negligible senescence in the longest living rodent, the naked mole-rat: insights from a successfully aging species, J. Comp. Physiol. B 178 (2008) 439–445.