Mutation in clock gene *period* increases susceptibility to oxidative stress in ageing *Drosophila melanogaster*

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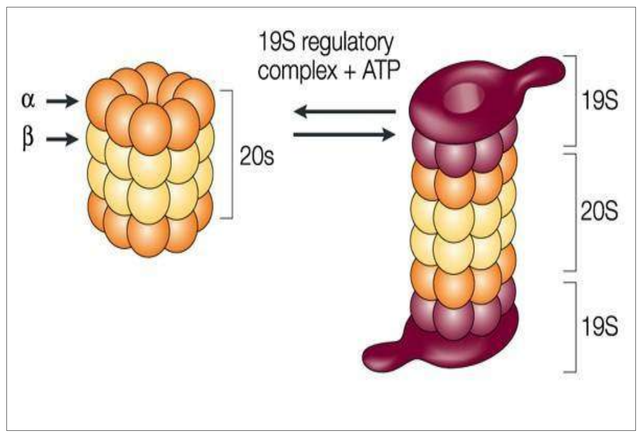
Introduction

Many organisms display daily rhythms in behavioral and biochemical processes. These cycles, known as circadian rhythms are entrained by several environmental factors, and continue even in a constant environment. In *Drosophila melanogaster*, known as the fruit fly, rhythmic expression of clock genes, such as *period*, occurs in the central nervous system and in many peripheral tissues [1]. Behavioral rhythms have been observed for many decades since they are easy to detect. Less is known about physiological rhythms and the adaptive value of circadian clocks. Understanding of these processes is vital to our comprehension of circadian rhythms and their significance for human health and fitness.

A recent study suggested that the circadian clock plays a crucial role in defense against oxidative stress [2]. Previous work in our lab demonstrated that mutant *Drosophila* that lacked a functional clock gene (*period*) was significantly more susceptible to protein damage compared to its wild-type counterpart. Oxidative stress has emerged as an important topic in studies involving in aging and age-related diseases due to the detrimental effects of reactive oxygen species (ROS) to the side chains of amino acid residues in specific proteins and enzymes in animals [3].



Studies involving protein damage and aging have shown that the rate of protein oxidation increases dramatically during the last third of an organism's life span [4]. This suggests that protein damage due to oxidative stress may accelerate aging in various organisms, including humans. Accumulation of damaged proteins and reduction in the activities of important proteases are known to alter the cellular integrity [5]. Carbonyl formation is an irreversible process and has been closely associated with aging. One of the most important factors controlling the levels of damaged proteins in an organism is the multi-enzymatic proteolytic complex called the proteasome. The proteasome is used to degrade damaged or misfolded protein by breaking the peptide bonds between the amino acid residues. Since the process of carbonylation is irreversible the only way a protein carbonyl can be degraded is by the proteasome. The specific proteasome particle that is of interest in this study is the 20S (shown in figure above), which serves as the catalytic core of the proteasome and has been implicated in elimination of oxidatively damaged proteins. The 20S particle is unique because it acts independently of ATP or ubiquitin markers, and it has three main proteolytic activities with specific functions to degrade oxidatively modified peptide sequences [5].The loss of 20S proteasome activity can result in the accumulation of oxidatively damaged protein and lead to many negative effects that occur during aging.

Materials and Methods

Fly rearing and strains: The wild-type strain, Canton-S (CS), and *period* mutant (*per*^{01}) flies [2] which do not produce PER protein were used in the study. The *per*-null flies were backcrossed to the CS flies six times to equalize the genetic background of both strains; isogenized wild-type flies were designated CS^{P}. Flies were reared on standard yeast-cornmeal-molasses diet. We designated time of day using the Zeitgeber Time (ZT) standard; by convention, ZT0 is the time of lights-on while ZT12 is the time of lights-off. All flies were reared in LD (12L:12D) from the egg stage at a constant temperature of 25°C. In all experiments only male flies were tested.

Hyperoxia administration: Four different groups of flies were aged to 5 days, 20 days, 35 days, and 50 days old. 25 male flies of both genotypes (CS^{P} and *per*^{01}) at different ages were exposed to 100% oxygen at a flow rate of approximately 300ml/min for 24 hours.

Levels protein carbonyls and Western blots : Total protein carbonyls were assayed as described previously (6). Western Blots for protein carbonyls were performed using OxyBlot protein oxidation detection kit (Chemicon International, USA).

20S proteasome activity assay: The 20S proteasome activity was assayed as described by [7] in the bodies from groups of 25 flies (from both genotypes) of different ages before and after hyperoxia.

Oxygen chamber used to treat flies with 100% O_2 with a flow rate of 300 ml/min for 24 hours.

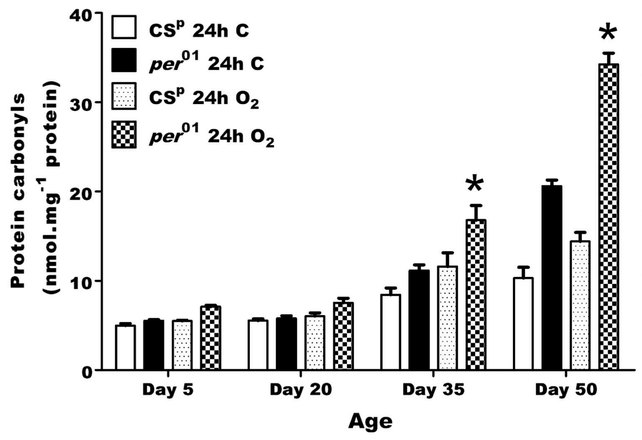
Peptidase activities of proteasome were assayed fluorimetrically in a Biotek Synergy II plate reader using specific substrates: Chymotrypsin-like activity - Suc-LLVY-AMC, Trypsin-like activity - BZVGR-AMC, and Caspase-like activity - Z-LLE-AMC (Biomol, USA).

References

[1] Nitabach, M.N. and Taghert, P.H. 2008.Organization of the *Drosophila* Circadian Control Circuit. Curr. Biol. 18: R84-R93.
[2] Krishnan, N. et al., 2008 .Circadian Regulation of Response to Oxidative Stress in *Drosophila melanogaster*. Biochem.Biophys. Res. Commun. 374: 299-303.
[3] Das, N. et al., 2001. Selectivity of Protein Oxidative Damage During Aging in *Drosophila melanogaster*. Biochem J. 360: 209-216.
[4] Beal, M. F. 2002. Oxidatively Modified Proteins in Aging and Disease. Free Radic. Biol. Med. 32: 797-803.
[5] Friguet, B. et al., 2000. Protein Degradation by the Proteasome and its Implications in Aging. Ann.N.Y. Acad. Sci. 908:143-154.
[6] Krishnan, N. et al., 2007. 20-Hydroxyecdysone Prevents Oxidative Stress Damage in Adult *Pyrrhocoris apterus*. Arch. Insect Biochem. Physiol. 65: 114-124.
[7] Kisselev, A.F. and Goldberg, A.L. 2005. Monitoring Activity and Inhibition of 26S Proteasomes with Fluorogenic Peptide Substrates. Methods. Enzymol. 398: 364-378.

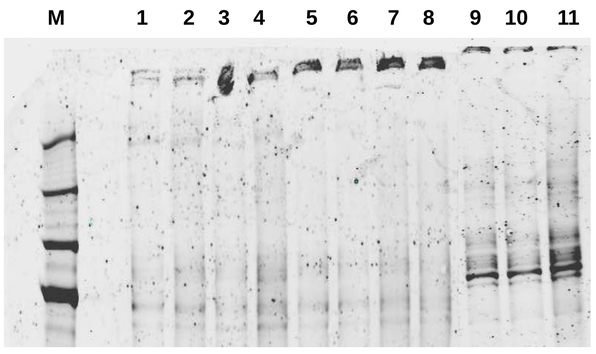
Results

Flies with disrupted circadian clock (*per*^{01}) accumulate more oxidative damage with increasing age.



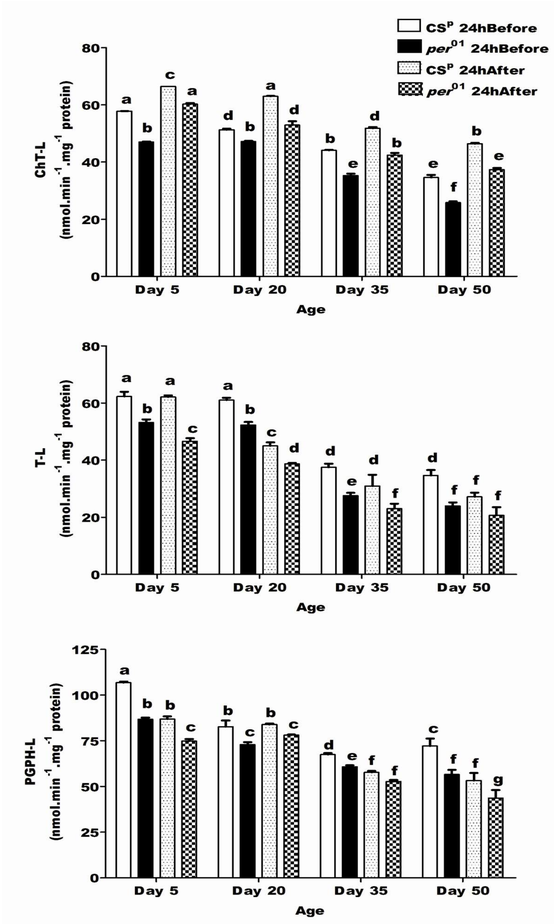
Protein carbonylation in the bodies of both the mutant and wild type flies increased as aging progressed. Age-dependent levels of protein carbonyl levels were significantly (* = p<0.001) higher in *per*^{01} flies than CS^{p} flies after 24h hyperoxia (O_2) on Day 35 or 50. Untreated (C) *per*^{01} flies also accumulated significantly (p<0.001) more carbonyls than CS^{p} on Day 35 and 50.

Western Blot- Immunoblotting of total proteins after derivatization with 2,4 Dinitrophenyl hydrazine followed by probing with specific antibody to DNPH revealed that mutant *per*^{01} flies after hyperoxia treatment had the greatest accumulation of carbonylated protein. The most intense bands of protein carbonyl groups correlated with a weight of ~40 kDa when compared with the standard marker. Markers 37-250 kDa in left lane. Starting from left: After marker, the samples are in sequence CS^{p} and *per*^{01} before and after hyperoxia with varying age.



1 = CS^{P} 24h B (Day 5) **5** = CS^{P} 24h B (Day 20) **9** = per^{01} 24h B (Day 35)
2 = per^{01} 24h B (Day 5) **6** = per^{01} 24h B (Day 20) **10** = CS^{P} 24h A (Day 35)
3 = CS^{P} 24h A (Day 5) **7** = CS^{P} 24h A (Day 20) **11** = per^{01} 24h A (Day 35)
4 = per^{01} 24h B (Day 5) **8** = per^{01} 24h B (Day 20)

Flies with disrupted clock (*per*^{01}) show accelerated decrease in 20S proteasome activity in bodies during aging and hyperoxia stress.



The overall 20S proteasome activity decreased with increasing age in both genotypes. The trypsin-like (T-L) activity decreased significantly (p<0.05) after 24 hyperoxia at each tested age. The caspase-like (PGPH-L) activity showed similar pattern of decreasing activity with age, but overall showed the highest activity. The chymotrypsin-like (ChT-L) activity showed interesting results wherein enzymatic activity actually increased after the flies had been exposed to the hyperoxia treatment.
In *per*^{01} flies both T-L and PGPH-L activities significantly declined with age and hyperoxia compared with their wild-type counterparts. ChT-L activity declined with age in both genotypes with greater decline in *per*^{01} flies but activity significantly increased following hyperoxia though the increase was significantly lesser in mutant flies. Bars with different superscripts are significantly different at p<0.05.

Conclusions

- Mutant *per*^{01} flies with a disrupted clock show increased protein carbonylation with increasing age, especially after oxidative challenge. This suggest that they may be more susceptible to ox stress.
- The 20S proteasome activity decreases with age in control CS flies. Exposure to hyperoxia accelerates the age-dependent loss of the 20S proteasome activity.
- Age-dependent decline in the activity of the 20S proteasome is exacerbated in mutant *per*^{01} flies with a disrupted clock.
- Our data suggest that the *period* gene is involved in the protection of an aging organism from oxidative damage by supporting the function of 20S proteasome.