RESEARCH ARTICLE

Low-level caloric restriction rescues proteasome activity and Hsc70 level in liver of aged rats

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Abstract Proteasome activity is known to decrease with aging in ad libitum (AL) fed rats. Severe caloric restriction (CR) significantly extends the maximum life-span of rats, and counteracts the age-associated decrease in liver proteasome activities. Since few investigations have explored whether lower CR diets might positively counteract the age associated decrease in proteasome activity, we then investigated the effects of a mild CR regimen on animal life-span, proteasome content and function. In addition, we addressed the question whether both CR regimens might also affect the expression of Hsc70 protein, a constitutive chaperone reported to share a role in the function of proteasome complex and in the repair of proteotoxic damage, and whose level decreased during aging. In contrast to severe CR, mild CR had a poor effect on life-span; however, it better counteracted the decrease of proteasome activities. Both regimens, however, maintain Hsc70 in liver of old rats at level comparable to that of young rats. Interestingly, the effects of aging and CRs on liver proteasome enzyme activities did not appear to be associated with parallel changes in the amount of proteasome proteins suggesting that the quality (molecular activity of the enzymes) rather than the quantity are likely to be modified with age. In conclusion, the results presented in this work show that a mild CR can have beneficial effects on liver function of aging rats because is adequate to counteract the decrease of proteasome function and Hsc70 chaperone level.

Abbreviations

AL Ad	Libitum
CT-L	Chymotrypsin-like
CR	Caloric Restriction
EOD	Every other day
FW	Fasted 1 day every week
Hsp70	Heat shock protein 70 kD
Hsc70	Heat shock cognate protein 70 kD
PGPH	Peptidoglutamyl-peptide-hydrolase or caspase-like
T-L	Trypsin-like

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Introduction

Accumulation of abnormal (oxidized, unfolded, cross-linked, glycated) proteins is known to occur

in mammalian cells during aging both in vitro and in vivo. Oxidatively damaged proteins may cross-link each other, form aggregates which can disrupt cellular homeostasis, and accumulate in cells and tissues and impair functioning (Harman 1981; Grune et al. 2001; Radak et al. 2002). In rat liver cells this phenomenon occurs at very old animal age, between 24 and 26 months (Starke-Reed and Oliver 1989; Vitttorini et al. 1999). Protein turnover is a main pathway for removal of damaged proteins; however, it may decline with age, thus contributing to their accumulation (Ward 2000). It has been suggested that an impairment of major proteolytic mechanisms such as proteasome and/or lysosome pathways may lead to the age-dependent failure of cell homeostasis (Holliday 1996; Cuervo and Dice 2000; Carrad et al. 2002; Chondrogianni et al. 2002; Hipkiss 2006).

Proteasome is a multimeric proteinase complex responsible for the degradation of abnormal, unfolded or oxidatively damaged proteins. In mammalian cells, 80–90% of protein degradation occurs via the proteasome pathway (Lee and Goldberg 1998). There is general agreement that proteasome activity declines during the process of aging in cultured cells and in a variety of tissues (Bulteau et al. 2000; Sitte et al. 2000; Gaczynska et al. 2001; Merker et al. 2001; Carrard et al. 2002). In human aging epidermis the decline in proteasome activity appeared to be related to down-regulation of purified proteasome subunits (Bulteau et al. 2000), and in aged human fibroblasts the impaired proteasome function was directly linked to the lower levels of a few β-catalytic subunits (Chondrogianni et al. 2003). In mice and rat liver proteasome peptidase activities decrease as a function of age (Conconi et al. 1996; Shibatani et al. 1996; Goto et al. 2002). Interestingly, in liver tissues the amount of proteasomes do not apparently change with age, suggesting a loss of enzyme activities without destruction of the inactivated enzyme molecules (Hayashi and Goto 1998; Goto et al. 2001; Goto et al. 2007).

It is now recognized that caloric restriction (CR) retards aging and extends life- and health-span in mammalian organisms, efficacy depending on duration and level (Masoro 2000). A restriction in food intake to 50–70% of that of the ad libitum (AL) fed rats or an intermittent (every-other day, EOD) feeding (Goodrick et al. 1983) markedly increase longevity, retard age-associated physiological deterioration, delay and, in some cases, prevent age-associated diseases (Masoro 2000). Milder (e.g. 10%) restrictions of the daily caloric intake may cause a smaller but still significant increase in longevity (Duffy et al. 2001). A low-frequency intermittent fasting (a 1-day/week fasting regimen) too may have significant anti-aging and anti-tumour effects in rodents (Cavallini et al. 2002; Berrigan et al. 2002). The underlying biological mechanisms responsible for life extension in caloric restricted animals are a matter of debate. Severe CR may increase the rate of protein turnover (Tavernarakis and Driscoll 2002) and retard the age-associated accumulation of oxidatively damaged molecules (Vittorini et al. 1999; Bokov et al. 2004); it may also increase lysosomal autophagic degradation (Bergamini et al. 2004) and proteasome activity (Shibatani et al. 1996; Vittorini et al. 1999; Gaczynska et al. 2001; Merker et al. 2001; Carrard et al. 2002). On the other hand, reduction of global protein synthesis may protect from oxidative stress and extended life span in Caenorhabditis elegans (Hipkiss 2007) independently of the mechanisms that involve insulin/IGF signalling and dietary restriction (Syntichaki et al. 2007). Severe CR may also restore muscle CT-L and PGPH activities to levels corresponding to their proper controls (Selsby et al. 2005). It has been reported that CR initiated in late adulthood of rats reverses the age-associated attenuation of proteasome activity in rat muscles and tendons (Radak et al. 2002) and in rat liver (Goto et al. 2002). Surprisingly, the effects of milder CRs on proteasome function have not been explored so far.

Chaperones such as heat shock proteins (Hsps) are ubiquitous, conserved proteins that assist folding of newly synthesized polypeptides or refolding of damaged proteins (Hartl 1996; Barral et al. 2004). Hsc70, the main constitutive member of the Hsp70 family, has been shown to share a role in the assembly of the 20S proteasome complex (Schmidtke et al. 1997). Moreover, Hsps favor the proteasomal degradation of selected proteins (Garrido and Solary 2003). Bag-1, a co-chaperone for Hsp70 and Hsc70, binds to the proteolytic machinery in an ATP-dependent manner, and CHIP, another co-chaperone for Hsc70-Hsp70 and Hsp90, tilts the folding-refolding machinery toward the degradative proteasome pathway (McDonough and Patterson 2003). These and other observations thus provide links and

cooperation between the chaperone system and the proteasome structure (Luders et al. 2000; Esser et al 2004). An increase in misfolded proteins with a simultaneous increase in chaperone occupancy, i.e., a "chaperone overload" may occur in aging organisms (Nardai et al. 2002). Overloading of the available chaperones may disturb the balance between chaperone requirement and function, limit the resources of protein folding, maintenance and turnover, and compromise general protein homeostasis and cellular function thus contributing to the aging process (Arslan et al. 2006; Sőti and Csermely 2003). Furthermore, proteasomal dysfunction associated with aging is mostly related to the inhibitory effect that accumulation of incompletely degraded and cross-linked proteins exerts on proteasome activity (Hipkiss 2006). The effects of a 30%-CR were investigated and results suggested that CR might counteract the age-associated changes in inducible and/or constitutive Hsp levels (Sőti and Csermely 2002).

The present study was designed to evaluate the effects of different CR diets on the overall survival and to compare the effects of these regimens on the activity of the three proteasome enzymes CT-L, T-L, and PGPH, and on the level of proteasome in liver of rats of different ages. The expression of the chaperone protein Hsc70, the main constitutive member of the Hsp70 family, was simultaneously studied by analyzing changes in its level.

Materials and methods

Animals and diet

The original founder stocks of the Sprague Dawley rats were obtained from Harlan Italy (S. Pietro al Natisone, Udine, Italy) and the breeding colony established from these animals has been maintained for 7 years in a conventional environment at the Center for Gerontological Research of the University of Pisa. The average body weight of rats in this colony has remained constant. The male rats that were used in this study were maintained at 20–22°C, and conditioned to a 12 h light/12 h dark cycle with lights on from 6:00 to 18:00 h daily. An ad libitum (AL) feeding regimen was used for all animals from the time they were weaned (at 3 weeks of age) until the time they entered the experimental protocol. The

animals were housed in standard rat cages, and received Teklad diet (Harlan Italy) and water ad libitum. At approximately 3 months of age, the test animals were separated into three groups: a control group that continued to receive food AL, and two CR groups which were fed either AL 6 days and fasted 1 day every week (FW) or fed AL every-other-day (EOD). On a weekly base, food consumption of FW rats was 90% and food consumption of EOD rats was 70% of the AL regimen. 40 animals of each group (AL, FW, EOD) were included in the following survival analysis that lasted for 21 months. Other groups of animals remained in their respective nutritional groups for only 9 months prior to sacrifice (for biochemical tests). Rats were fed (or food was withdrawn) at 14:00 h daily, which corresponded to 8 h after lights on. On the day of ad libitum feeding, rats were offered more food than they could consume in 24 h. Weight of withdrawn food was recorded and food consumption was computed by the difference. Food was withdrawn 16 h before experimentation. Rats on the EOD restricted diet were sacrificed on the day of fasting. Rats were anaesthetized by the intraperitoneal injection of pentobarbital (50 mg/kg body weight), the right liver lobe was taken for experimentation. The excised organ was plunged in liquid nitrogen and stored at –80°C.

Determination of proteasome activity

Frozen livers were minced and homogenized in ice-cold buffer (20 mM Tris-HCl pH 7.5, containing 10% glycerol, 5 mM ATP and 0.2% NP-40) using an homogenizer and then frozen and thawed three times. Membrane, cellular debris and mitochondria were eliminated by centrifuging at 12,000 x g for 10 min at 4°C. Protein concentrations were determined by Bio-Rad protein-assay (Bio-Rad Laboratories, Richmond, CA, USA).

Proteasome activity was assayed according to the procedure described in detail elsewhere (Bonelli et al. 2004). The three proteasome activities (chymotrypsin-like (CT-L), trypsin-like (T-L), and peptidoglutamyl-peptide-hydrolase (PGPH)) were assayed in Proteasome Assay Buffer, containing 25 mM Hepes pH 7.5, 0.03% SDS, 0.05% NP-40, with 50 μM fluorogenic peptide substrate, after 1 h of incubation at 37°C. The assay of CT-L and T-L activities is based on the

detection of the fluorophore, 7-amino-4-methylcouma-rin (AMC) after cleavage from the labeled substrates Suc-Leu-Leu-Val-Tyr-AMC and Boc-Leu-Arg-Arg-AMC, respectively. The assay of PGPH activity is based on the detection of the fluorophore, β-naphthyl-amine (βNA) after cleavage from the labeled substrate Z-Leu-Leu-Glu-βNA. The free AMC and βNA fluo-rescence were quantified using a 380/460 and a 337/430 nm filter set respectively in a LS 50 B lumines-cence spectrometer (Perkin-Elmer, Fremont, CA, USA). Proteasome activity was calculated as the difference between the total activity of tissue extracts and the remaining activity in the presence of 20 μM of the proteasome inhibitor MG132.

Western blotting

Frozen livers were minced and homogenized in ice-cold buffer (10 mM NaCl, 3 mM MgCl$_2$, 10 mM Tris-HCl pH 7.4, 0.1% SDS, 0.1% Triton, 10 μg/ml APMSF, 0.5 μg/ml Leupeptin, 0.7 μg/ml Pepstatin, 0.5 mM EDTA) using an homogenizer. The homog-enate was then centrifuged at 12,000 x g for 10 min at 4°C, and protein concentrations of tissue extracts were determined by Bio-Rad protein-assay.

Protein analysis by 1-D PAGE is described in detail elsewhere (Petronini et al. 1989; Petronini et al. 1993). Immunoblotting was carried out as previously described (Petronini et al. 1993; Bonelli et al. 2001) according to the method of Burnette (1981), using a polyclonal antibody directed against 20S proteasome α/β subunits (Biomol Inter., Plymouth Meeting, PA, USA), and a monoclonal antibody directed against actin (Sigma-Aldrich, St. Louis, MO, USA). Monoclonal antibody directed against constitutive Hsc70 (N27F3-4) was a generous gift from Dr. W.J. Welch (San Francisco, CA, USA).

The bands corresponding to proteasome subunits, Hsc70 and actin were detected on autoradiography films using the ECL system, according to the manufacturer's protocol (Millipore Corp., Billerica, MA, USA). The intensities of the bands were quantified using a densitometer (UN-SCAN-IT gel, Automated Digitizing System, Silk Scientific Corp., Orem, UT, USA) and the data were expressed as integrated intensity (units of optical density x volume) of the bands.

Statistical analysis

Data are expressed as mean ± standard deviation. One-way ANOVA, and Dunnet's test for post hoc analysis were used. P values of 0.01–0.05 were considered as significant (**$P < 0.01$, *$P < 0.05$). The significant P values higher than 0.05 and less than 0.10 indicated a trend.

To analyze the effects of diets, estimates of the survival probability were calculated using the Kap-lan-Meier method, and the log rank test was employed to test the null hypothesis of equality in overall survival among groups.

Results

Effect of caloric restriction on life span

The differences between the survival curves for AL, FW and EOD rats were analyzed by log-rank statistics, and the analysis indicates a significant difference between the survival probabilities of the three groups ($P = 0.001$), each group consisting of forty animals. As shown in Fig. 1, the difference of surviving probabil-ities occurred only between the AL group and the EOD group ($P = 0.00033$) with 17 deaths out of 40 versus 3 deaths out of 40 animals. The other two curves analyzed (AL vs. FW and EOD vs. FW) showed only a trend ($P = 0.0597$ and $P = 0.0652$, respectively) with 9 deaths out of 40 for the FW group.

Effect of aging and caloric restriction on proteasome activity and level

Proteasome enzymatic activities (CT-L, T-L, PGPH) were measured in the post-mitochondrial surnatant extracts of liver obtained from 3-, 12- and 24-month-old rats. As shown in Table 1, the analysis of 12-month-old rats showed a significant decrease in CT-L activity in liver extracts in comparison with extracts obtained from 3-month-old animals. Similarly, PGPH activity was also significantly reduced, while T-L activity did not change. Analysis of 24-month-old rats evidenced a further marked decline in both CT-L and PGPH activities. Otherwise, T-L activity signif-icantly decreased only in 24-month-old rat liver.
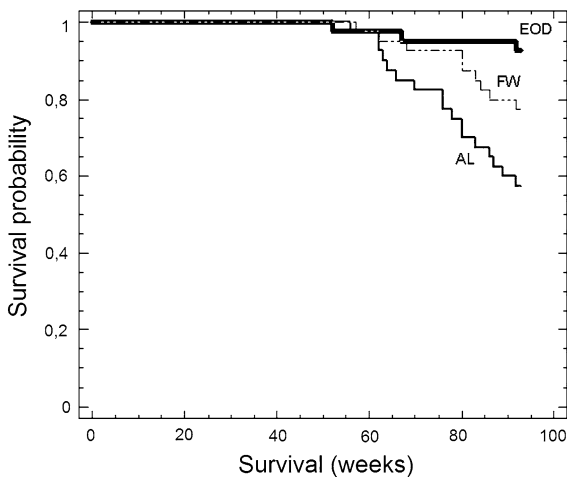
Fig. 1 Survival curves for male Sprague-Dawley rats fed ad libitum (AL), mild CR (FW) and severe CR (EOD). Curves are generated from 40 rats for each group. Non-parametric estimates of survival were calculated from the start of the different (AL, FW, EOD) CR treatments (13th week). The pair-wise log-rank statistics gave the following results: AL versus FW, P-value = 0.0597; AL versus EOD, P-value = 0.00033; FW versus EOD, P-value = 0.0652

The effects of two different types of caloric restriction, mild and severe, were then examined in rats of different ages. As shown in Fig. 2, 24-month-old rats exposed to a mild caloric restriction (FW) had increased CT-L and PGPH proteasome activities in comparison to animals of the same age but fed ad libitum (AL). Interestingly, a significant effect of the mild diet on PGPH activity was already evident in the liver of 12-month-old rats. However, 24-month-old animals exposed to a severe CR did increase significantly PGPH activity, meanwhile the diet effect on CT-L activity indicated only a trend (P = 0.058).

Table 1 Decline of proteasome activity with aging

	3 Months	12 Months	24 Months
CT-L	75.1 ± 4.6	50.4 ± 4.6**	40.8 ± 2.9**
T-L	103.7 ± 5	106 ± 3.8	91.3 ± 0.8*
PGPH	140 ± 13.2	86.7 ± 0.6**	72.3 ± 10.4**

CT-L, T-L and PGPH activities of the proteasome were measured on tissue extracts of liver from 3-, 12- and 24-month-old rats as described in Materials and Methods. The proteasome activities are expressed as arbitrary fluorescence units per 50 μg of proteins. Results are given as mean ± SD of three measurements of n = 4 rats in each group. *P < 0.05 and **P < 0.01, compared with the proteasome activities of 3-month-old rats

On the whole, the results so far obtained indicated that mild CR rather than severe CR prevented the decline in CT-L and PGPH activities in the liver of aging rats. In contrast, T-L activity, which only declined slightly in the liver of 24-month-old animals, was not significantly affected by mild or severe CR.

The decreased enzyme activities might be related to a general slowing down of protein turnover during aging, and to a reduced content in proteasomes. A Western blot analysis was performed by using a polyclonal rabbit antisera against 20S core proteasome α/β subunits. This demonstrated that the level of the 20S proteasome did not change as a function of age or after caloric restriction (Fig. 3).

Effect of aging and caloric restriction on Hsc70 level

Since we hypothesized that during aging changes in the expression of chaperone Hsp might coincide with the decrease of proteasome activity, and with the accumulation of damaged proteins, the amounts of the constitutive chaperone Hsc70 was determined for all our experimental conditions. By the use of a monoclonal antibody directed against the constitutive Hsc70, a Western blotting analysis showed that the level of Hsc70 was significantly reduced in the liver of both 12 and 24 month old rats in comparison with young rats (see Fig. 4). The decrease in the level of this prominent chaperone protein might reflect either the decline in protein synthesis rate with aging as well as a proteotoxic damage with a consequent increased rate of Hsc70 degradation, not adequately replaced by newly synthesized molecules. Interestingly, both mild and severe CR were fully adequate to maintain in the liver of aging rats the expression of Hsc70 at the same level of that observed in the liver of 3-month-old rats.

Discussion

The main objective of this study was to find out if a mild regimen of CR prevents age-related changes on the three enzyme (CT-L, T-L, and PGPH) activities associated with the proteasome function. It is known

Fig. 2 Effect of aging and of caloric restriction on liver proteasome activity. CT-L, T-L and PGPH activities of the proteasome were measured on tissue extracts of liver from young (3-month-old), adult (12-month-old) and old (24-month-old) rats. Both adult and old animals were fed ad libitum (AL, □), severe (EOD, ■) and mild caloric restricted (FW, ▨). Each bar of the histogram represents the percentage of proteasome activity versus control (3-month-old rats). The values of the controls corresponded to 75.1 ± 4.6 (CT-L), 103.7 ± 5 (T-L) and 140 ± 13.2 (PGPH) arbitrary fluorescence units/50 μg of proteins (cf Table 1). Results are given as mean ± SD of three measurements of $n = 4$ rats in each group. $*P < 0.05$ and $**P < 0.01$, compared with the control of the same age
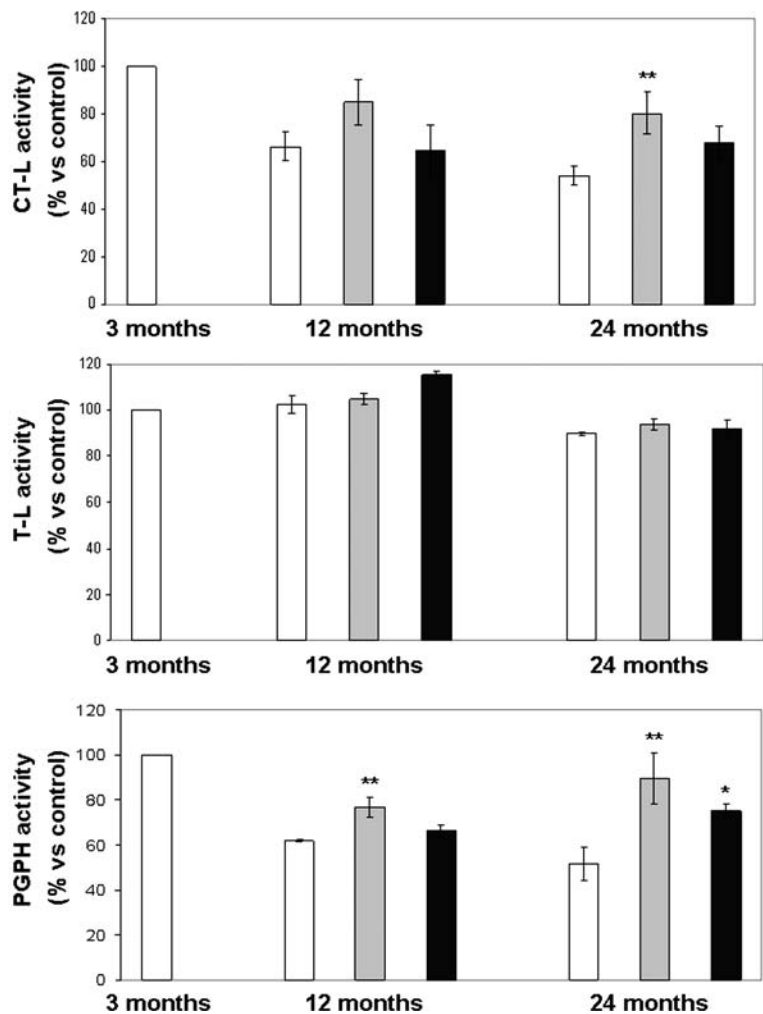
that proteasome activity decreases in the liver of old rats (Hayashi and Goto 1998; Goto et al. 2001; Goto et al. 2002) as well as in other organs and tissues (Keller et al. 2000; Radak et al. 2002; Selsby et al. 2005). In keeping with these results, in our group of animals we found that in comparison with young animals proteasome activity was significantly reduced in 12-month-old animals and further decreased in 24-month-old rats. The exposure of rats to life long mild CR almost fully counteracted the aging-associated decrease of CT-L and PGPH proteasome activities. Moreover, the age-dependent decrease in the level of the chaperone protein Hsc70, the main constitutive chaperone having an important role in the repair of proteotoxic damage and likely to be involved in proteasome assembly, was fully counteracted by mild CR.

The attenuation of proteasome activities during aging might be explained by at least two mechanisms that are not mutually exclusive: (a) a reduced expression of proteasome subunits due to the inability of an aged cell, overloaded by altered proteins, to eliminate them and/or to cope with an unbalanced protein turnover, and (b) the inactivation of proteasome enzymes by alteration (for example, oxidative damage and/or glycation) of their structure during aging. Immunoidentification of proteasome levels by polyclonal antibody indicated that the amount of overall proteasomes did not change with age in liver of animals either fed ad libitum or exposed to CR. Considering that in in human fibroblasts (Chondrogianni et al. 2003) a decreased level of specific proteasome β-subunit activity and expression has been reported, the possibility that in the liver of aging
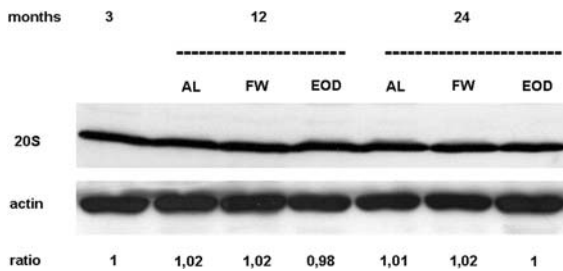
Fig. 3 Expression of α/β subunits of 20S proteasome in liver of AL, FW and EOD CR rats. Cells proteins were extracted from liver of young (3-month-old), adult (12-month-old) and old (24-month-old) animals fed ad libitum (AL), severe caloric restricted (EOD) and mild caloric restricted (FW). Total proteins were then separated by SDS-PAGE, blotted onto nitrocellulose, and treated with a polyclonal antibody directed against α/β subunits (20S proteasome) or actin. The ratio represents the result of a densitometric quantitation of 20S proteasome level, normalized to actin. Similar results were obtained in experiments performed with three rats in each group
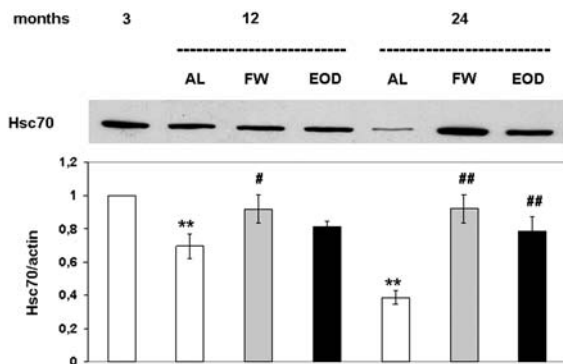

Fig. 4 Expression of Hsc70 in liver of AL, FW and EOD CR rats. Cells proteins were extracted from liver of young (3-month-old), adult (12-month-old) and old (24-month-old) animals fed ad libitum (AL, □), severe caloric restricted (EOD, ■), mild caloric restricted (FW, ▨). Total proteins were then separated by SDS-PAGE, blotted onto nitrocellulose, and treated with a monoclonal antibody directed against the constitutive Hsc70. Similar results were obtained in three different experiments. Histogram represents the result of a densitometric quantitation of Hsc70 level, normalized to actin, and the values are the mean $\pm$ SD of three experiments performed with three rats in each group. $**P < 0.01$, compared with control (3-month-old) animals; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with the control animals of the same age

rats a decreased expression of some β-subunits might occur (and not revealed by the polyclonal antibody used by us) can not be excluded. However, when proteasome activity is investigated in tissues,

divergent results occur: for instance, in rat diaphragm, in contrast to locomotor skeletal muscle (Radak et al. 2002), level and function of key proteasome components and ubiquitin-conjugating enzyme activity are preserved during senescence (Kavazis et al. 2007). Moreover, it has been reported that in rat liver changes in activities with aging and CR are not accompanied by change in the amount of purified proteasome as detected by antiproteasome antisera (Shibatani et al. 1996) or by antibodies against subunits of the enzymes (Goto et al. 2007). These and our results, indicating that the activity of the enzymes are modified with age and/or CR without apparent change of proteasome level, are also consistent with the idea of chemical or structural alterations in proteasomes (Goto et al. 2002). In this context, a recent observation of an age-related increase in glycated $\alpha7$ subunit of the proteasome in human cells undergoing aging in vitro indicated that it might be exploited as a marker of proteasomal malfunction (Gonzales-Dosal et al. 2006).

Only a few authors have analyzed the effect of a severe CR on proteasome activities. For instance, PGPH activity appeared to recover in liver of rats exposed to a severe CR, while T-L and CT-L activities did not change following CR (Shibatani et al. 1996). Moreover, the T-L activity of both soleus and gastrocnemius muscle (Radak et al. 2002; Selsby et al. 2005) appeared to recover in rats when exposed to a severe CR. Our results show that milder CR is adequate to maintain proteasome activities by counteracting their decline. Furthermore, our data show that the effect of a mild CR regimen on PGPH activity is already evident in 12-month-old rats animals in comparison to 3-month-old control rats.

The positive role of recovery of proteasome function by CR is supported by recent studies showing that increasing proteasome content extends yeast lifespan and enhanced viability during oxidative stress (Chen et al. 2006). Furthermore, overexpression of proteasome accessory protein in human fibroblasts has been reported to enhance proteasome-mediated antioxidant defence (Chondrogianni and Gonos 2007).

In regard to antiaging intervention, although dietary antioxidants have been extensively used as a model, by far the best results have been obtained with severe CR diets started early during the life of the organism. They have been shown to lead to a substantial reduction in disease morbidity, with an

increased life-span (Fernandes et al. 1997; Masoro 2000). Understanding the mechanisms by which CR exerts its benefits may aid design of more acceptable anti-aging strategies, which might also reduce obesity and age-related diseases such as cancer (Greenwald et al. 2001; Roth et al. 2002). We found here that although the mild CR shows only a tendency to influence longevity, it appeared better than the severe CR for maintaining Hsc70 expression and proteasome activities of old animals at the level of young controls. This suggests that the maximum rate of proteasome proteolysis might not be a limiting factor of longevity and that the frequency of stimulation might have an important role in maintaining optimal protein turnover, which is consistent with a role of hormesis in aging (Rattan 2004) akin to the beneficial effects resulting from the cellular responses to repeated mild stress.

Recent data obtained in yeast cells, suggest that reduced protein synthesis rate, one of the most energy-consuming cellular process, positively affects lifespan. Under unfavorable conditions reduction of protein synthesis rates would result in energy savings. This energy could be diverted to repairing and maintenance processes, thus contributing to prolong lifespan. Similarly, elimination of a specific initiation factor 4E (eIF4E) reduces protein synthesis and extends lifespan in the nematode Caenorhabditis elegans (Tavernarakis 2007). At present, however, we cannot distinguish whether the beneficial effects of mild CR on rat liver proteasome function may be due to either reduced protein synthesis per se or to reduced levels of damaged proteins or both. Moreover, the possibility that CR induces the expression of chaperone-like Hsps to rescue the altered structure of proteasome enzymes and to counteract the formation of inhibitory cross-linked proteins (Carrard et al. 2002) has been advanced. Accordingly, it has been previously showed that the addition of Hsp90 to purified proteasome fractions protected T-L activity from inactivation by oxidative stress (Conconi and Friguet 1997). A subsequent study reported a reduced chaperone capacity of liver cytosol extracted from aged rats compared to those of young counterparts; although Hsc70/Hsp70 levels were not significantly different in livers of young and aged rats, old animals showed a significant decrease in their hepatic Hsp90 content (Nardai et al. 2002). Our results highlighted a significant decrease in Hsc70 level during aging.

Furthermore, following mild CR we noted a higher level of Hsc70 in the livers of aged rats associated with recovery of proteasome activity. The rescue of Hsc70 level is in agreement with some reports indicating elevated protein synthesis and turnover in response to CR (Tavernakis and Driscoll 2002). Interestingly, in rat muscle tissue following CR, Hsps have been found to be largely responsible for the restoration of the T-L activity of the proteasome (Selsby et al. 2005). Taken together with our finding that mild CR counteracts the decline of Hsc70, the rescue of this chaperone protein might be associated with the maintenance of proteasome activity (Chondrogianni and Gonos 2005). In conclusion, our results support the hypothesis that changes in Hsp expression would coincide with maintenance of proteasome activity, enhanced disposal of damaged proteins accumulated during aging, and improvement of liver functions.

References

Arslan MA, Csermely P, Sőti C (2006) Protein homeostasis and molecular chaperones in aging. Biogerontology 7:383–389

Barral JM, Broadley SA, Schaffar G, Hartl FU (2004) Roles of molecular chaperones in protein misfolding diseases. Semin Cell Dev Biol 15:17–29

Bergamini E, Cavallini G, Donati A, Gori Z (2004) The role of macroautophagy in the ageing process, anti-ageing intervention and age-associated diseases. Int J Biochem Cell Biol 36:2392–2404

Berrigan D, Perkins SN, Haines DC, Hursting SD (2002) Adult-onset calorie restriction and fasting delay spontaneous tumorigenesis in p53-deficient mice. Carcinogenesis 23: 817–822

Bokov A, Chaudhuri A, Richardson A (2004) The role of oxidative damage and stress in aging. Mech Age Dev 125:811–826

Bonelli MA, Alfieri RR, Desenzani S, Petronini PG, Borghetti AF (2004) Proteasome inhibition increases HuR level, restores heat-inducible HSP72 expression and thermotolerance in WI-38 senescent human fibroblasts. Exp Gerontol 39:423–432

Bonelli MA, Alfieri RR, Poli M, Petronini PG, Borghetti AF (2001) Heat-induce proteasomic degradation of HSF1 in serum-starved human fibroblasts aging in vitro. Exp Cell Res 267:165–172

Bulteau AL, Petropoulos I, Friguet B (2000) Age-realted alterations of proteasome structure and function in aging epidermis. Exp Gerontol 35:767–777

Burnette WN (1981) Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to humidified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 112:195–203

Carrard G, Bulteau AL, Petropoulos I, Friguet B (2002) Impairment of proteasome structure and function in aging. Int J Biochem Cell Biol 34:1461–1474

Cavallini G, Donati A, Gori Z, Parentini I, Bergamini E (2002) Low level dietary restriction retards age-related dolichol accumulation. Aging Clin Exp Res 14:152–154

Chen Q, Thorpe J, Dohmen J, Li F, Keller JN (2006) Ump1 extends yeast life span and enhances viability during oxidative stress: central role for the proteasome? Free Radic Biol Med 40:120–126

Chondrogianni N, Fragoulis EG, Gonos S (2002) Protein degradation during aging: the lysosome-, the calpain- and the proteasome-dependent cellular proteolytic systems. Biogerontology 3:121–123

Chondrogianni N, Gonos ES (2005) Proteasome dysfunction in mammalian aging: Steps and factors involved. Exp Gerontol 40:931–938

Chondrogianni N, Gonos ES (2007) Overexpression of hUMP1/POMP protease accessory protein enhances protease-mediated antioxidant defence. Exp Gerontol 42:899–903

Chondrogianni N, Stratford FLL, Trougakos IP, Friguet B, Rivett AJ, Gonos ES (2003) Central role of the proteasome in senescence and survival of human fibroblasts: induction of a senescence-like phenotype upon its inhibition and resistance to stress upon its activation. J Biol Chem 278:28026–28037

Conconi M, Friguet B (1997) Proteasome inactivation upon aging and on oxidation-effect of hsp90. Mol Biol Rep 24:45–50

Conconi M, Szweda LI, Levine RL, Stadtman ER, Friguet B (1996) Age-related decline of rat liver multicatalytic proteinase activity and protection from oxidative inactivation by heat-shock protein 90. Arch Biochem Biophys 331:232–240

Cuervo AM, Dice JF (2000) When lysosomes get old. Exp Gerontol 35:119–131

Duffy PH, Seng JE, Lewis SM, Mayhugh MA, Aidoo A, Hattan DG, Casciano DA, Feuers RJ (2001) The effects of different levels of dietary restriction on aging and survival in the Sprague-Dawley rat: implications for chronic studies. Aging (Milano) 13:263–272

Esser C, Albert S, Höhfeld J (2004) Cooperation of molecular chaperones with the ubiquitin/proteasome system. Biochim Biophys Acta 1695:171–188

Fernandes G, Venkatraman JT, Turturro A, Hart RW (1997) Effect of food restriction on life span and immune functions in long-lived Fischer-344 x Brown Norway F1 rats. J Clin Immunol 17:85–95

Gaczynska M, Osmulski PA, Ward WF (2001) Caretaker or undertaker? The role of the proteasome in aging. Mec Age Dev 122:235–254

Garrido C, Solary E (2003) A role of HSPs in apoptosis through "protein triage". Cell Death Differ 10:619–620

Gonzales-Dosal R, Sorensen MD, Clark BFC, Rattan SIS, Kristensen MD (2006) Phage-displayed antibodies for the detection of glycated protease in aging cells. Ann NY Acad Sci 1067:474–478

Goodrick CL, Ingram DK, Reynolds MA, Freeman JR, Cider NL (1983) Differential effects of intermittent feeding and voluntary exercise on body weight and lifespan in adult rats. J Gerontol 38:36–45

Goto S, Takahashi R, Araki S, Nakamoto H (2002) Dietary restriction initiated in late adulthood can reverse age-related alterations of protein and protein metabolism. Ann NY Acad Sci 959:50–56

Goto S, Takahashi R, Kumiyama A, Radak Z, Hayashi T, Takenouchi M, Abe R (2001) Implications of protein degradation in aging. Ann NY Acad Sci 928:54-64

Goto S, Takahashi R, Radak Z, Sharma R (2007) Benefit biochemical outcomes of late-onset dietary restriction in rodents. Ann NY Acad Sci 1100:431–441

Greenwald P, Clifford CK, Milner JA (2001) Diet and cancer prevention. Eur J Cancer 37:948–965

Grune T, Shringarpure R, Sitte N, Davies K (2001) Age-related changes in protein oxidation and proteolysis in mammalian cells. J Gerontol A Biol Sci Med Sci 56:B459–B467

Harman D (1981) The aging process. Proc Natl Acad Sci USA 78:7124–7128

Hartl F-U (1996) Molecular chaperones in cellular protein folding. Nature 381:571–579

Hayashi T, Goto S (1998) Age related changes in the 20S and 26S proteasome activities in the liver of male F344 rats. Mech Age Dev 102:55–66

Hipkiss AR (2006) Accumulation of altered proteins and aging: causes and effects. Exp Gerontol 41:464–473

Hipkiss AR (2007) On why decreasing protein synthesis can increase lifespan. Mech Age Dev 128:412–414

Holliday R (1996) Understanding ageing. Cambridge University Press, Cambridge

Kavazis AN, DeRuisseau KC, McClung JM, Whidden MA, Falk DJ, Smuder AJ, Sugiura T, Powers SK (2007) Diaphragmatic protease function is maintained in the ageing Fisher 344 rat. Exp Physiol 92:895–901

Keller JN, Hanni KB, Markesbery WR (2000) Possible involvement of proteasome inhibition in aging: implication for oxidative stress. Mech Age Dev 113:61–70

Lee DH, Goldberg AL (1998) Proteasome inhibitors cause induction of heat shock proteins and trehalose, which confers thermotolerance in Saccharomices cerevisiae. Mol Cell Biol 18:30–38

Luders J, Demand J, Hohfeld J (2000) The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. J Biol Chem 275:4613–4670

Masoro EJ (2000) Caloric restriction and aging: an update. Exp Gerontol 35:299–305

McDonough H, Patterson C (2003) CHIP: a link between the chaperone and proteasome systems. Cell StressChaperones 8:303–308

Merker K, Stolzing A, Grune T (2001) Proteolysis, caloric restriction and aging. Mech Age Dev 122:595–615

Nardai G, Csermely P, Söti C. (2002) Chaperone function and chaperone overload in the aged. A preliminary analysis. Exp Gerontol 37:1257–1262

Petronini PG, Alfieri R, De Angelis EM, Campanini C, Borghetti AF, Wheeler KP (1993) Different HSP70

expression and cell survival during adaptive responses of 3T3 and transformed 3T3 cells to osmotic stress. Br J Cancer 67:493–499

Petronini PG, Tramacere M, Mazzini A, Kay JE, Borghetti AF (1989) Control of protein synthesis by extracellular Na$^+$ in cultured fibroblasts. J Cell Physiol 140:202–211

Radak Z, Takahashi R, Kumiyama A, Nakamoto H, Ohno H, Ookawara T, Gono S (2002) Effect of aging and late onset dietary restriction on antioxidant enzymes and proteasome activities, and protein carbonylation of rat skeletal muscle and tendon. Exp Gerontol 37:1423–1430

Rattan SIS (2004) Aging, anti-aging and hormesis. Mech Ageing Dev 1125:285–289

Roth GS, Lane MA, Ingram DK, Mattison JA, Elahi D, Tobin JD, Muller D, Metter EJ (2002) Biomarkers of caloric restriction may predict longevity in humans. Science 297:811

Schmidtke G, Schmidt M, Kloetzel PM (1997) Maturation of mammalian 20S proteasome: purification and character-ization of 13S and 16S proteasome precursor complexes. J Mol Biol 268:95–106

Selsby JT, Judge AR, Yimlamai T, Leeuwenburgh C, Dodd SL (2005) Life long calorie restriction increases heat shock proteins and proteasome activity in soleus muscles of Fisher 344 rats. Exp Gerontol 40:37–42

Shibatani T, Nazir M, Ward WF (1996) Alteration of rat liver 20S proteasome activities by age and food restriction. J Gerontol 51:B316–B322

Sitte N, Merker K, von Zglinicki T, Grune T (2000) Protein oxidation and degradation during proliferative senescence of human MRC-5 fibroblasts. Free Radic Biol Med 28:701–708

Sőti C, Csermely P (2002) Chaperones come of age. Cell Stress Chaperones 7:186–190

Sőti C, Csermely P (2003) Aging and molecular chaperones. Exp Gerontol 38:1037–1040

Starke-Reed PE, Oliver CN (1989) Protein oxidation and proteolysis during aging and oxidative stress. Arch Bio-chem Biophys 275:559–567

Syntichaki P, Troulinaki K, Tavernarakis N (2007) eIF4E function in somatic cells modulates ageing in Caeno-rhabditis elegans. Nature 445:922–926

Tavernarakis N, Driscoll M (2002) Caloric restriction and lifespan: a role for protein turnover? Mech Ageing Dev 123:215–229

Tavernarakis N (2007) Protein synthesis and aging. Cell Cycle 6:1168–1171

Vittorini S, Paradiso C, Donati A, Cavallini G, Masini M, Gori Z, Pollera M, Bergamini E (1999) The age-related accu-mulation of protein carbonyl in rat liver correlates with the age-related decline in liver proteolytic activities. J Gerontol A Biol Sci Med Sci 54:B318–323

Ward WF (2000) The relentless effects of the aging process on protein turnover. Biogerontology 1:195–199