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Mild heat stress stimulates 20S proteasome and its 11S activator in human fibroblasts undergoing aging in vitro

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Abstract Repeated mild heat shock (RMHS) has been shown to have several beneficial hormetic effects on human skin fibroblast undergoing aging in vitro. Because an age-related decline in proteasome activity is 1 of the reasons for the accumulation of abnormal proteins during aging, we have investigated the effects of RMHS on the 20S proteasome, which is the major proteolytic system involved in the removal of abnormal and oxidatively damaged proteins. Serially passaged human skin fibroblasts exposed to RMHS at 41°C for 60 minutes twice a week had increased 3 proteasomal activities by 40% to 95% in early- and midpassage cultures. RMHS-treated cells also contained a 2-fold higher amount of the proteasome activator 11S, and the extent of the bound activator was double in early- and midpassage cells only. Furthermore, there was no difference in the content of the 19S proteasome regulator in the stressed and the unstressed cells. Therefore, RMHS-induced proteasome stimulation in early- and midpassage fibroblasts appears to be due to an induction and enhanced binding of 11S proteasome activators. In contrast to this, the proteasomal system in late-passage senescent cells appears to be less responsive to the stimulatory effects of mild heat shock.

INTRODUCTION

In recent years, various approaches have been developed to modulate aging at cellular and organismic levels. One of these approaches involves the application of mild stress as a stimulant of intracellular maintenance and repair processes, with beneficial antiaging and longevity promoting effects, known as hormesis (Minois 2000; Rattan 2001). Using the Hayflick model system of cellular aging of serially passaged normal diploid cells in vitro (Hayflick 1965), our group has reported a variety of hormetic effects by repeated mild heat shock (RMHS) in human skin fibroblasts. It was found that although RMHS did not affect the replicative lifespan of fibroblasts, RMHS-treated cells had reduced rate and extent of several senescence-related characteristics, including reduced cell enlargement, reduced accumulation of oxidized and glycoxidized abnormal proteins, increased ratio of reduced and oxidized glutathione, increased resistance to sugar-induced damage to protein, and an increased basal level of various heat shock proteins (Hsp) (Rattan 1998; Verbeke et al 2001a, 2002; Fonager et al 2002). Because a reduction in the accumulation of oxidized and glucoxidized proteins was the major effect observed in these studies, the current study was undertaken to ascertain whether the RMHS-treated cells have an improved degradation mechanism of damaged or abnormal proteins.

Progressive accumulation of abnormal and aggregated proteins is 1 of the most widely reported age-related changes. In particular, oxidized, glycoxidized, and ubiquitinated proteins have been shown to accumulate in an age-related fashion in various organisms (Oliver et al 1987), in cells undergoing aging in culture (Verbeke et al 2001a; Chondrogianni et al 2002, 2003), and in certain pathologies, such as Alzheimer's and Parkinson's diseases (Keller et al 2000; McNaught and Jenner 2001). Furthermore, the fact that there is a reciprocal relationship between the rate of protein turnover and aging (Chondrogianni et al 2000; Grune 2000; Sitte et al 2000b; Ward

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2002; Carrard et al 2003) indicates that the protein degradation pathways are impaired with age. There are several proteolytic systems partaking in the intracellular proteolysis of these damaged and abnormal proteins, where the major systems are the proteasome/ubiquitin (Ub) - proteasomal proteolytic pathway, the calpains associated with the cytoskeleton, and the lysosomal proteolytic pathways (Cuervo and Dice 1998; Chondrogianni et al 2002). Oxidized proteins, though, are preferentially degraded by the 20S proteasome in an adenosine triphosphate (ATP)–independent manner (Grune 2000; Davies 2001), whereas the proteins marked by covalently attached Ub are degraded in an ATP-dependent way by the 26S proteasome (Ciechanover et al 2000b; Pickart 2001). Calpain is a calcium-dependent protease and is mainly responsible for the elimination of cytoskeletal and membrane proteins (Kawasaki and Kawashima 1996). The lysosomes are organelles that are taking care of most of the nonspecific protein degradation and also are responsible for the incessant basal turnover of many long-lived proteins and chaperone-mediated autophagy (Cuervo and Dice 2000).

The proteasome is a multisubunit, multicatalytic proteinase complex, also known as multicatalytic proteinase, and is ubiquitous among eukaryotes and archaea (Zwickl 2002). The eukaryotic proteasome is present both in the nucleus and in the cytoplasm where some of it is associated with the endoplasmic reticulum membrane (Rivett 1998) and with the cytoskeleton (Scherrer and Bey 1994). It constitutes approximately 1% of the total content of cytosolic protein (Tanaka et al 1986). The substrates for the proteasome fall into 2 categories: (1) misfolded, denatured, or otherwise damaged proteins and (2) perfectly normal proteins, which have to be removed for normal function of the cell, such as cell cycle control, protein quality control, apoptosis, and antigen presentation. Hydrolysis of amide bonds by the proteasome is indispensable for essentially all vital cellular processes, and there is an intricate regulation of its cellular activities (DeMartino and Slaughter 1999; Ciechanover et al 2000a).

Besides being the central processing unit, the 20S proteasome in eukaryotic cells also serves as a proteolytic core of 2 larger proteasomal assemblies: the 19S regulator (PA700) and the 11S activator (PA28 or REG). These molecules bind to both ends of the proteasome and modify its proteolytic properties. When the 19S regulator binds to both ends of the 20S proteasome, the complex is called the 26S proteasome and has an estimated molecular weight of approximately 2100 kDa (DeMartino and Slaughter 1999). The identification of the 19S regulator as the cap of the 26S proteasome complex is based on its subunit composition (DeMartino et al 1994) and on its ability to stimulate Ub-dependent protein degradation (Coux et al 1996). The 11S proteasome activator is a hexa- or heptameric ring structure that stimulates the peptidase activity of the 20S proteasome. The 11S activator cannot recognize and degrade ubiquitinated substrates and it, therefore, has been hypothesized that the 11S activator acts as an adapter molecule between 20S proteasome and cytosolic chaperones (Stolzing and Grune 2001). Abnormalities in intracellular proteolysis are widespread in aging, and age-related alterations in the proteasomal system, including a decreased activity of the proteasome toward artificial peptide substrates as well as the ability to degrade oxidized proteins during aging, have been reported (Conconi et al 1996; Sitte et al 2000b, 2000c; Chondrogianni et al 2003).

In this study, we report the results of our studies on the effects of RMHS on the 3 activities of the proteasome and the contents of proteasomal components, including 11S activator and 19S regulator.

MATERIALS AND METHODS

Cell culture and mild stress treatment

Primary cultures of normal human diploid skin fibroblasts, designated ASF-1, were established by explant growth from a mammary epithelial biopsy from a 30-year-old, healthy, female donor as described previously (Verbeke et al 2001a). Cells were grown at 37°C, 5% CO_2 and 95% humidity, in 75 cm^2 flasks (COSTAR, Cambridge, MA, USA) in 14 mL of Dulbecco modified Eagle medium (Bio Whittaker, Verviers, Belgium) containing 10% (vol/vol) European grade fetal calf serum (Biological Industries, Beit Haemek, Israel), 1% (vol/vol) glutamine, and 1% (vol/vol) penicillin-streptomycin. Monolayer cell cultures were split at confluence as follows, while keeping a record of the input and output of number of cells, using a Model Z-2-Coulter Counter (Ramcon, Birkroed, Denmark). The split ratio at early stages was 1:4 until the growth rate slowed down and, therefore, was kept at 1:2 for the rest of the replicative lifespan. Cell cultures were serially passaged until the end of the replicative lifespan and the cumulative population doubling level (CPDL) determined, as described before (Verbeke et al 2001a). In this series of experiments, ASF-1 cells exhausted their proliferative capacity after attaining CPDL40, which was designated as 100% lifespan completed. For heat shock (HS) treatment, cultures were exposed to RMHS at 41°C for 60 minutes twice a week throughout their lifespan, as described previously (Verbeke et al 2001a). The cells exposed to RMHS in this series of experiments had received HS 104 times and attained CPDL40, similar to unstressed controls.

Sample collection and immunoblotting

Protein extraction for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and nondenatur-

ing-PAGE was prepared by collecting the cells as described previously (Fonager et al 2002). Cell pellets were resuspended in phosphate-buffered saline containing protease inhibitors (1 Complete™ tablet; Roche Diagnostics GmbH, Schwetzingen, Germany) in 50 mL with 5 mM ethylenediamine-tetraacetic acid (EDTA) and disrupted by sonication (4 pulses of 30 seconds, with a 20-second break in between) while kept on ice. Membranes and cellular debris were removed by centrifugation at 15 000 × *g* for 30 minutes at 4°C. Protein content in the supernatant was determined using the Bradford method (Bio-Rad, Hercules, CA, USA). The supernatant was stored at −80°C. SDS-PAGE was conducted using 12% or 15% acrylamide (wt/vol) separations gels (Laemmli 1970). Nondenaturing-PAGE was done using a 5% acrylamide (wt/vol) gel, a method modified from Hedrick and Smith (1968). Immunoblot experiments were performed after SDS-PAGE separation of 20 μg total cellular proteins, followed by electrotransfer into Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The primary antibody used to investigate the total proteasome population was a rabbit anti-20S proteasome polyclonal antibody (Conconi et al 1996), kindly provided by Dr Bertrand Friguet. The antibody against 11S was a polyclonal anti-PA28 alpha antibody (Affinity Bioreagents, CO, USA). The anti-19S antibody was a polyclonal antibody against the S2 subunit (Affinity Bioreagents, CO, USA), and the one against actin was a monoclonal anti–β-actin antibody (Sigma, Copenhagen, Denmark). Detection of bands was carried out after incubation with secondary antibody (DAKO, Glostrup, Denmark) as described previously (Fonager et al 2002). Analysis of the bands on the autoradiograph was performed using Lotus Graphic software. β-Actin was used to internally correct the measurements. Enzyme-linked immunosorbent assay (ELISA) was performed using a monoclonal antibody against the α2 subunit of the 20S proteasome, a gift from Dr Bertrand Friguet.

Proteasome activity assays

A cytosolic extraction for the proteasome activity assay was performed by collecting cell pellet by scraping off the cells. Cell pellet was homogenized in 300 μL of an extraction buffer (50 mM NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid [HEPES] pH 8, 0.5 M sucrose, 1 mM EDTA, 0.2% [vol/vol] Triton X-100, 1 M phenylmethane sulfonyl fluoride, 25 μL pure β-mercaptoethanol). Cells were disrupted by sonication (4 pulses of 30 seconds) while kept on ice. Membranes and cellular debris were removed by centrifugation at 15 000 × *g* for 30 minutes at 4°C. The supernatant was stored at −80°C, and thawing-freezing steps were avoided. For Chymotrypsinlike (ChT-L) activity and peptidylglutamyl-peptide hydrolase (Caspaselike [C-L]) activity, 20 μg of cellular protein extract was incubated with the suc-LLVY-AMC (where AMC refers to 7-amido-4-methylcoumarin) peptide (Sigma) (to a final concentration of 25 μM in HEPES buffer [0.1 M, pH 8.0]) and the Cbz-LLE-β-NA (where NA refers to naphthylamide) peptide (Sigma) (to a final concentration of 25 μM in HEPES buffer), respectively, in a final volume of 200 μL. The mixture was incubated for 60 minutes at 37°C, and the reaction was stopped by adding 300 μL of acidic solution (30 mM sodium acetate, 70 mM acetic acid, 100 mM sodium chloroacetate, pH 4.3) and 300 μL of ice-cold ethanol, respectively. Trypsinlike (T-L) activity was determined by incubating 50 μg of protein extract with 40 μM *N*-*t*-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (LSTR-AMC) (Sigma) to a final volume of 200 μL in the HEPES buffer. The mixture was incubated at 37°C for 90 minutes, and the reaction was stopped by adding 300 μL of acidic solution. The solutions were adjusted to 3 mL with distilled water to fill the polymethacrylate fluorimeter cuvettes (Sigma). All activity measurements were done in triplicate from independent samples, and the fluorescence of cleavage products from peptide substrate was measured on a Kontron SFM 25 spectrofluorimeter. AMC, the product of the suc-LLVY-AMC and the Boc-LSTR-AMC peptides, was detected at 350 nm excitation and 440 nm emission wavelengths. The excitation and emission wavelengths were 333 and 410 nm, respectively, for β-NA, the product of the Cbz-LLE-β-NA peptide. The proteasome activity was expressed in terms of nanomolars of AMC/β-NA detected on the fluorometer per minute of incubation time per milligram of cytosol protein used in the assay. For each activity measured, a negative control was performed by using 10 μL of a solution of the proteasome inhibitor MG132 (Sigma) at a final concentration of 400 μM that was incubated with each sample, and proteasome activities were measured as described above. Final proteasome activity was determined as the difference between the total activity and the remaining activity of the crude extract in the presence of the proteasome inhibitor MG132.

Separation of different proteasome complexes by gel filtration

Cells were lysed in 20 mM Tris-HCl buffer, pH 7.5, and the lysate was centrifuged at 11 500 × *g* for 10 minutes at 4°C. Gel filtration was carried out using a Superose 6 column (Rivett et al 2002). The column was equilibrated in 20 mM Tris-HCl buffer, pH 7.5, containing 10% glycerol, 5 mM ATP, and 100 mM NaCl, which are the buffer conditions for separating different proteasome complexes (Bose et al 2001). The soluble cell extracts loaded on the Superose 6 column, each contained 2.0 mg protein. From

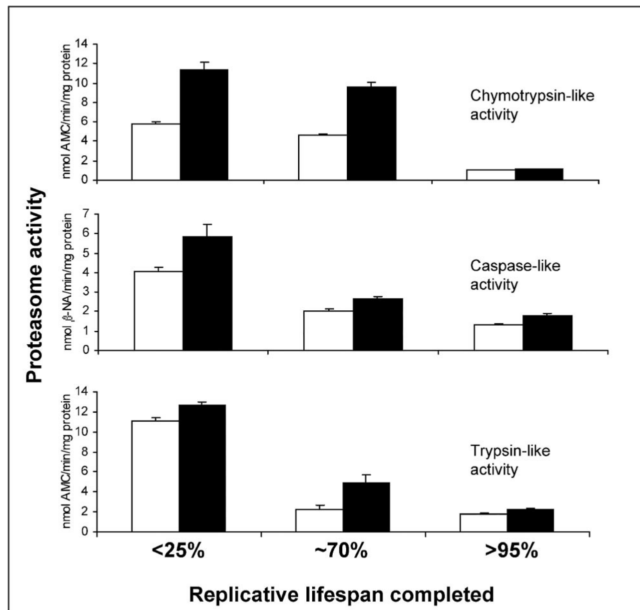

Fig 1. Three main activities of the proteasome in different age groups of serially passaged human skin fibroblasts with and without repeated mild heat shock (RMHS) treatment. Open bars are untreated control cells, and filled bars represent cells exposed to RMHS. All activities of the RMHS cells are measured 24 hours after the most recent HS. HS, heat shock.

the fraction of 0.5 mL each, 20 μL was taken for assaying the activity of different proteasome complexes, using the suc-LLVY-AMC peptide, as described above. The rest of the fraction was used for SDS-PAGE and immunoblot analysis. The presence of unbound 11S proteasome activator in the fractionated cell extracts was assayed by comparing the activity in the absence and in the presence of small amounts of purified 20S proteasome from human lymphocytes (Carrard et al 2003), kindly provided by Dr Bertrand Friguet. This assay was carried out in 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 1 mM β-mercaptoethanol for assembling of the 11S activator unit and the 20S proteasome (Bose et al 2001).

RESULTS

Proteasomal activities during aging and RMHS

ChT-L–, C-L–, and T-L–specific activities of the proteasome were detected in serially passaged adult human skin fibroblasts ASF-1 with and without exposure to RMHS. A comparison of the 3 age groups (early-passage young group with less than 25% replicative lifespan completed; midpassage group with about 70% replicative lifespan completed; and late-passage senescent cells with more than 95% lifespan completed) shows that there was a significant age-related decline in all the 3 main activities of the 20S proteasome (Fig 1). The ChT-L activity in the control (C) cells decreased by 20% from young to midage group of cells, whereas in the late-passage cells it was reduced by more than 80%. For the C-L activity in the C cells (Fig 1), there was a 50% decline in the midage group as compared with the young group. In the senescent C cells, the C-L activity further declined to a level that was 69% less than that in the young C cells. In a similar fashion, T-L activity of the proteasome in the C cells declined by 79% and 84% in the midage and senescent cells, respectively.

Following our protocol of RMHS, ASF-1 cells in this series of experiments received up to 104 HS during their lifespan. In cells exposed to RMHS, the proteasome activities were significantly higher in young and midage groups but not in late-passage senescent cells (Fig 1). In the young RMHS-treated cells, the ChT-L activity was almost 2-fold higher than that in the young C cells. During serial passaging, the activity declined by 14% in the midage cells and by 90% in the late-passage senescent cells. The C-L activity of the proteasome in the young cells exposed to RMHS was more than 30% higher than that in the C cells (Fig 1). During serial passaging, C-L activity of RMHS-treated cells decreased by 55% in the midage group and by 68% in the senescent cells, which was still 28% higher than that in C cells. For the T-L activity, the pattern was somehow different from that for the 2 other main activities. For example, in the young RMHS-treated cells, the T-L activity was about 14% more than the T-L activity of the proteasome in the young C cells, which declined by 62% in the midage group and by 80% when the cells reached the end of the replicative lifespan (Fig 1).

Proteasome and proteasome activators' content

To determine whether the age-related and RMHS-related differences in proteasomal activities could be due to variations in the proteasomal content, immunoblot and ELISA were used to detect the 20S proteasome population. Figure 2A shows the Western blot–estimated content of 20S proteasome in cell lysates prepared from both RMHS-treated and C cells in the 3 different age groups of serially passaged ASF-1 cells, correlated with the content of β-actin. There was no alteration in the content of combined subunits of the 20S proteasome, as detected by the polyclonal antibody used in this study (Fig 2A). The a-subunits content of the 20S proteasome was further investigated by ELISA (Fig 2B) for the similar age groups, and it is apparent that the content of the α-subunit from the 20S proteasome did not change significantly through the replicative lifespan of ASF-1 cells, with or without RMHS exposure.

We also investigated the levels of the proteasome activators 11S and 19S in early- and late-passage ASF-1 cells. Figure 3A shows the basal level of 11S content in the young RMHS-treated cells, which was twice as high as

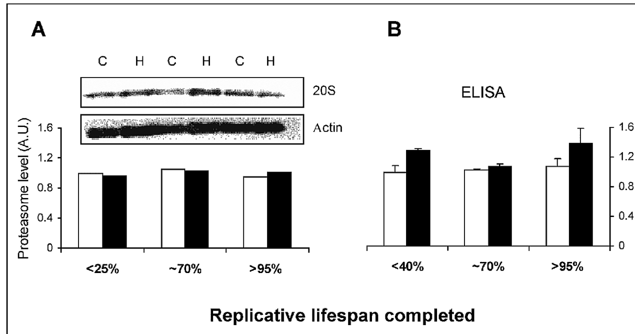

Fig 2. The 20S proteasome level in 3 age groups of serially passaged human skin fibroblasts with and without repeated mild heat shock (RMHS) treatment. (A) The immunoblot performed by using a rabbit anti-20S proteasome polyclonal antibody and a polyclonal antibody against β-actin. The histogram illustrates the 20S proteasome level after the content has been correlated with the content of β-actin. Open bars: untreated control cells, denoted C. Filled bars: cells exposed to RMHS, denoted H. The proteasome level is in arbitrary units (AU), taking the intensity of the young control cells (<25% lifespan completed) as 1 AU. The sample from the RMHS cells was prepared 24 hours after the most recent HS. (B) Histogram based on the results of an ELISA performed by using a monoclonal antibody against the α2 subunit of the 20S proteasome. Cells have here completed less than 40%, approximately 70%, and more than 95% of their replicative lifespan, respectively. ELISA, enzyme-linked immunosorbent assay; HS, heat shock.

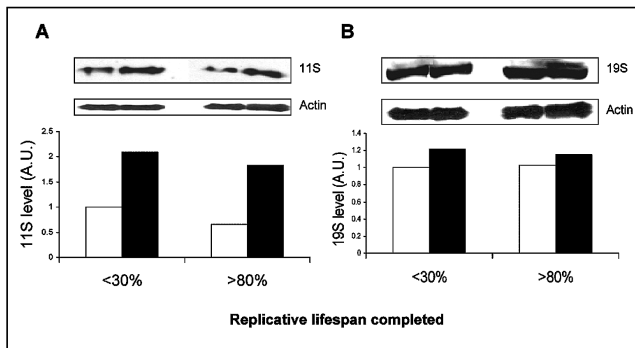

Fig 3. The level of 11S proteasome activator in early- and late-passage human skin fibroblasts with and without repeated mild heat shock (RMHS) treatment. (A) Immunoblot was performed by using a polyclonal antibody against the proteasome activator PA28 α-subunit. (B) Immunoblot performed by using a polyclonal antibody against the proteasome regulator 19S S2-subunit. The bars show the proteasome activator/regulator levels after the content was correlated with the content of actin from immunoblots made by using a polyclonal antibody against β-actin. Open bars: untreated control cells. Filled bars: cells exposed to RMHS. The levels are in arbitrary units (AU) taking the intensity of the early-passage control cells (30% lifespan completed) as 1 AU. Late-passage senescent cells have completed >80% of their lifespan.

that in the young C cells. In late-passage cells, the content of the 11S proteasome activator was reduced by 35% in the C cells but to a lesser extent (15%) in the RMHS-treated cells; therefore, the difference in the content of the 11S proteasome activator between C and RMHS cells was well maintained during cellular passaging (Fig 3A). Furthermore, we also had checked out the content of the 19S

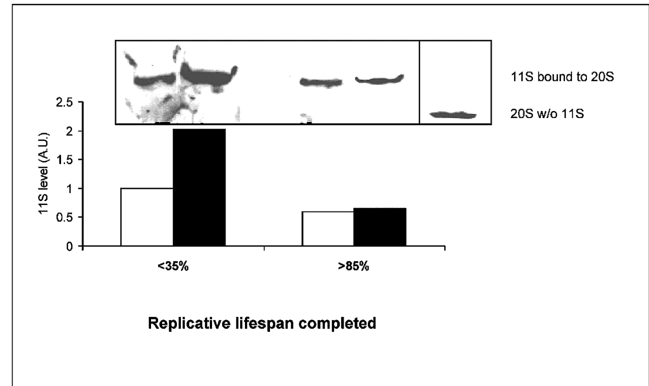

Fig 4. The amount of 11S activator units bound to the 20S proteasome. Immunoblot from a native gel, performed by using a polyclonal antibody against the proteasome activator PA28 α-subunit for the young and old cells, is shown on the left. Anti-20S proteasome polyclonal antibody was used as a control for migration, shown on the right. The histogram shows the amount of the 11S activators bound to the 20S proteasome. Open bars are untreated control cells, and filled bars represent cells exposed to repeated mild heat shock (RMHS). The data are in arbitrary units (AU) taking the intensity of the early-passage C-cells (<35% lifespan completed) as 1 AU. Late-passage senescent cells have completed >85% lifespan. The unbound 20S band is from early-passage (<35% lifespan completed) C cells. C, control.

proteasome regulator by immunoblot but did not find any significant differences in early- and late-passage C cells and in RMHS-treated cells (Fig 3B).

Because the proteasome activities in the late-passage C and RMHS-treated cells were not considerably different, but the level of the 11S proteasome activator was twice as high in the cells exposed to RMHS, we investigated the extent of 11S bound to the 20S proteasome. When 20S proteasome is not bound to any activators or regulators, it has a smaller size and therefore it migrates further down the native gel in comparison with when 11S is bound to the 20S proteasome. In Figure 4, it is shown that there was almost a 2-fold difference in the number of 11S activator units bound to the 20S proteasome in the young RMHS-treated cells as compared with untreated cells. However, in late-passage senescent cells, the variation between C cells and RMHS-treated cells was negligible, and both groups showed a highly reduced binding of the 11S activator to the 20S proteasome. The reason for the absence of the unbound 11S proteasome activator further down the gel is that the percentage of the native gel was so low (4%) that the small 11S unit was migrated out of the gel and was therefore not visible.

The analysis of the distribution of proteasome complexes in C cells and RMHS-treated cells was further carried out by chromatography of cell lysates on a Superose 6 column. Peak activities of 26S and 20S proteasomes eluted in fractions 23 and 28, respectively, demonstrated that for both RMHS-treated and C cells the majority of the proteasome complex was due to the 26S proteasome, as

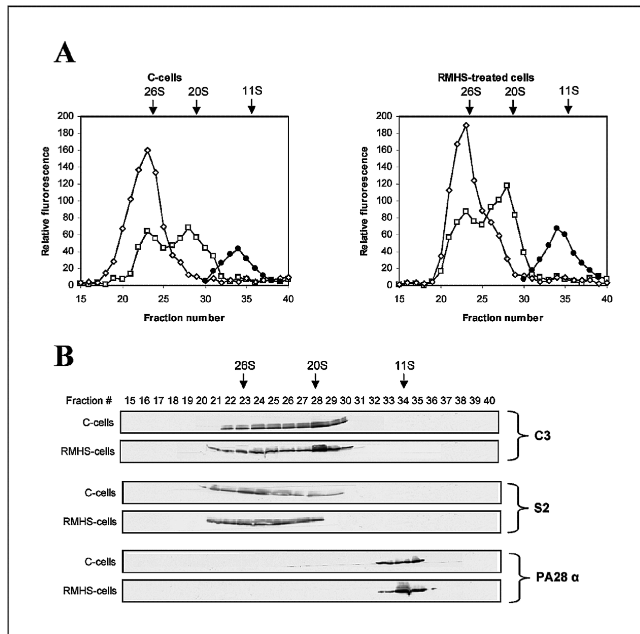

Fig 5. The separation of 26S and 20S proteasomes together with the 11S proteasome activator. (A) Extracts from control cells and repeated mild heat shock (RMHS)-treated cells (approximately 50% lifespan completed) were separated by chromatography. Arrows indicate the elution position of the 26S (◊) and 20S (□) proteasome complexes together with the 11S proteasome activator (●). (B) Immunoblot analysis of the different fractions with antibodies: Anti-C3 against 20S proteasome, anti-S2 against 19S proteasome regulator, and anti-PA28 α against 11S proteasome activator.

indicated by the arrows in Figure 5A. Furthermore, immunoblots in Figure 5B show that there was an almost even distribution between 26S and 20S proteasome fractions in the control. However, when cells were treated with RMHS, there was a shift in the distribution of the 20S proteasome. Here, the ratio of the C3 subunit in the 26S proteasome fractions was lower than that in the fractions containing the 20S proteasome. Using the anti-S2 antibody, it was shown that the decrease in the 26S proteasome level in the RMHS-treated cells was not accompanied by an increase in the free 19S units (Fig 5B). Both the 26S and the 20S proteasome activities were higher in the RMHS-treated cells, which was mainly due to their higher contents (Fig 5A).

The fractions from the Superose 6 column were also analyzed with anti-PA28 a antibody for the content of the 11S proteasome activator (Fig 5B). For both types of cells, the 11S activator eluted in fractions 33–35, with a peak in fraction 34. Figure 5B shows that there was a considerably higher amount of 11S units in the RMHS-treated cells than in the C cells. To confirm and verify the actual presence and functionality of the 11S proteasome activator, fractions containing free 11S activator units were preincubated with small amounts of exogenous 20S proteasome and were assayed in buffer conditions described in Materials and Methods. The results showed an approximately 2-fold increase in the 11S activator level in the 11S-containing fraction from RMHS-treated cells as compared with the C cells (Fig 5A), which is consistent with the data from the immunoblots (Fig 5B) and the data shown in Figure 4.

DISCUSSION

We have presented novel data that human fibroblasts exposed to mild heat stress at 41°C have significantly enhanced the 3 main proteasomal activities, which may account for the reduced levels of oxidized and glycoxidized proteins in such cells, reported by us previously (Verbeke et al 2000, 2001a, 2002). Earlier, another study had reported that rat macrophage activator (RMA) cells exposed to severe HS at 42°C had a reduction in ChT-L activity of the 20S proteasome (Kuckelkorn et al 2000). This highlights the difference between the biological consequences of severe and mild stress. In a series of reports published by us, including the present study, we have presented data on the beneficial effects of repeated mild stress on serially passaged human fibroblasts undergoing aging and senescence in culture (Rattan 1998; Verbeke et al 2001a, 2001b, 2002; Fonager et al 2002), and this phenomenon is known as hormesis (Minois 2000; Rattan 2001; Calabrese and Baldwin 2003). In contrast to this, severe stress is almost always harmful for biological systems, as exemplified by numerous reports on the effects of oxidative, thermal, and other stresses, which, in the case of serially passaged normal cells or immortalized cells, generally lead to premature cell cycle arrest, excessive telomere loss, rapid senescence, and death by apoptotic and necrotic pathways (Toussaint et al 2000, 2002; von Zglinicki 2002). As a result of these differences in the biological effects of severe and mild stress, applications of hormesis in clinical and antiaging research and therapy are being increasingly realized and explored (Rattan 2001; Calabrese and Baldwin 2003).

Another aspect of the hormetic effects of RMHS reported in our studies is the observation that whereas various cellular and biochemical characteristics of mild stress–exposed cells may improve in early- and midpassage cells, these alterations do not normally interfere with the genetically regulated replicative lifespan. In the present study too, it is clear that although RMHS-treated cells have higher proteasomal activities in early- and midage groups, this difference almost disappears in late-passage senescent cells where the plethora of numerous other changes can overshadow the improvement in 1 or a few characteristics. It will be interesting to find out whether combining different kinds of stresses, which involve different pathways of maintenance and repair, for example, DNA repair, antioxidants, HS response, and ly-

sosomal activation, will have cumulative or synergistic hormetic effects (or both). Such studies are potentially useful ultimately in developing practical methods to modulate aging and to improve the quality of life in old age (Rattan 2001).

Regarding the molecular mechanisms of stimulation of proteasomal activities, these remain to be elucidated. One pathway that has been studied in this respect is the association of the so-called 11S proteasome activator (Whitby et al 2000). For example, oxidative stress, which has also been reported to stimulate the activity of purified 20S proteasome from chicken red blood cells, is reported to bring about this effect by enhanced binding of the 11S proteasome activator (Strack et al 1996). Stimulation of the proteasome also has been observed in purified proteasome from lobster muscles treated with 11S (Mykles 1996) and in proteasome from rabbit skeleton muscles exposed to continuous motor nerve stimulation (Ordway et al 2000). In the present study, we have found that the RMHS treatment raises the basal level of the 11S proteasome activator. When comparing the 11S content in the 2 groups of young cells, it is remarkable that the pattern is similar to proteasome activities that also are significantly higher in the young RMHS-treated cells as compared with the young C cells. It has previously been reported by other groups that the association of the 11S activator with the 20S proteasome results in an elevation of the peptidase activities, as assayed by the fluorogenic peptides used to examine the specific activities of the proteasome (Dubiel et al 1992; Ma et al 1992; Hoffman and Rechsteiner 1996; Kuehn and Dahlmann 1996, 1997; Rechsteiner et al 2000). It, therefore, is possible that RMHS treatment does not activate the proteasome directly, but instead, induces the synthesis of 11S proteasome activators. The association of the 11S activator with the 20S proteasome then leads to the stimulation of the proteolytic activities of the proteasome.

It has previously been claimed that the activity of the 26S proteasome decreases when exposed to heat and other kinds of stress (Orino et al 1991; Peters et al 1994). This makes it even more plausible that the explanation for the RMHS stimulation of the proteasome is mainly by the induction and binding of the 11S proteasome activator to the 20S proteasome. Another possibility for the stimulation of 20S proteasomal activity is an increase in the content of 19S, when cells are exposed to stress (Mykles 1989; Ordway et al 2000). In the RMHS-treated cells, there is a higher level of 26S proteasome, but the distribution of the 20S proteasome is still highest toward exogenous 20S proteasome. Thus, the RMHS-treated cells have higher 26S and 20S proteasome contents as compared with the untreated C cells. However, this does not explain the observed decrease in RMHS-treated senescent cells where the activity of the proteasome is almost the same as for the C cells. Even though the antibodies used in this study did not reveal an age-related decline in the total proteasome content, it has recently been reported that there is a selective decline in only the 3 proteolytic β-subunits, and not in any of the other subunits, in human fibroblasts undergoing aging in vitro (Chondrogianni et al 2003). It would be interesting to find out whether a similar selective stimulation or loss of proteasomal subunits (or both) occurs in RMHS-treated cells during aging. Other reasons for the age-related loss in proteasomal activities include the accumulation of oxidized proteins, which have been reported to inhibit the proteasome activity (Sitte et al 2000a; Bulteau et al 2001), or an alteration in the structure and composition of the 20S proteasome subunits (or both) in the senescent cells. Further studies are required to fully understand the exact mechanisms for the loss of proteasomal activities during aging.

The observation that the 3 main proteasome activities are being stimulated when cells are exposed to RMHS and the finding that the 11S proteasome activator is induced by RMHS treatment raise some interesting possibilities. For example, when normal cells are exposed to mild HS, the 11S proteasome activators, which are present in the cells at a basal level, bind to the proteasome during the HS and thereby stimulate the activity. During the HS and in the following duration, more 11S activator units are transcribed and translated, thereby continuously causing an increase in the proteasome activity in the hours after the HS. Furthermore, Hsp are synthesized during and after HS, and some of those Hsp are suggested to accelerate the peptidase activity of the proteasome when the 11S activator is attached to it because the proteasome activator is functioning as an adaptor molecule between the 20S proteasome and the Hsp (Stolzing and Grune 2001). We have previously reported that cells treated with mild HS throughout their lifespan have a higher basal level of Hsp70, Hsp27, and heat shock cognate 70 (Fonager et al 2002), as is the case for the content of 11S activator units reported in the present study. Thus, it appears that higher levels of both the Hsp and the number of 11S units bound to the 20S proteasome in RMHS-treated cells make it possible for them to activate the proteasome rapidly when exposed to mild stress, resulting in the observed beneficial effects in cells.

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