Free Radical Biology & Medicine, Vol. 31, No. 12, pp. 1593–1602, 2001

0891-5849/01/$–see front matter

PII S0891-5849(01)00752-3

Original Contribution

REDUCED LEVELS OF OXIDIZED AND GLYCOXIDIZED PROTEINS IN HUMAN FIBROBLASTS EXPOSED TO REPEATED MILD HEAT SHOCK DURING SERIAL PASSAGING IN VITRO

PHILIPPE VERBEKE, BRIAN F. C. CLARK, and SURESH I. S. RATTAN

Danish Centre for Molecular Gerontology, Laboratory of Cellular Ageing, Department of Molecular and Structural Biology, University of Aarhus, Aarhus, Denmark

(*Received* 7 *August* 2001; *Accepted* 27 *September* 2001)

Abstract—Repeated mild heat shock (RMHS) has beneficial hormesis-like effects on various characteristics of human skin fibroblasts undergoing replicative senescence in vitro. We have tested whether RMHS could reduce the accumulation of oxidized and glycoxidized proteins, which is a major age-related change. Levels of carbonylated proteins, furosine, Nε-carboxymethyl-lysine-rich proteins and advanced glycation end products increased during serial passaging of fibroblasts in culture. However, the extent of accumulation of oxidized and glycoxidized proteins was significantly reduced in RMHS cells. The basal concentration of reduced glutathione was higher and that of oxidized glutathione was lower in RMHS cells. Whereas the basal level of heat shock protein HSP27 decreased in both RMHS and control cells during serial passaging, the increase of the basal level of HSP70 with increasing passage level was significantly higher in RMHS cells. These results show that the slower accumulation of damaged proteins in fibroblasts exposed to RMHS results partly from the increased ability of these cells to cope with oxidative stress, and to synthesize HSP responsible for protein capping and refolding. © 2001 Elsevier Science Inc.

Keywords—Replicative senescence, Hormesis, Oxidation, Glycoxidation, Heat shock response, Stress, Anti-aging, Fibroblast, Free radicals

INTRODUCTION

Protein oxidation and protein glycation/glycoxidation are two of the well-known posttranslational modifications occurring progressively during aging [1–5]. These modifications are both due to an imbalance of the cellular redox status leading to increased oxidative stress and due to alterations in enzymatic pathways related to redox state. Protein oxidation is mainly due to the attack of radical oxygen species (ROS) [1] on amino acids, generating oxo-, sulfo-, hydroxy-, chloro-, and nitro-derivatives [2,6]. Several amino acids may yield a common modification to form carbonyl derivatives, which is often used as a marker of protein oxidation [2]. On the other hand, protein glycation is due to the nonenzymatic reaction of sugars or of metabolic products of sugars, amino acids, ascorbate, and lipids, with the free amine of a lysine or arginine residues [4,7]. The Maillard pathway, due specifically to sugar attachment, leads to the formation of an early glycation product called Amadori product (AP) [8]. AP, as well as highly reactive α-oxoaldehydes produced from other metabolic pathways, can undergo a complex series of reactions to form the so-called advanced glycation end products (AGE) [7,9,10]. In addition, the advanced glycoxidation end products are generated by sequential glycation and metal-catalyzed oxidation reactions, to form products such as Nε-carboxymethyl-lysine (CML) and pentosidine [11–13]. Moreover, AP and glycoxidation products themselves produce ROS, reinforcing the oxidative stress and promoting the synergistic involvement of oxidation and glycation/glycoxidation processes [9].

All biological systems have a variety of defense mechanisms to counteract the effects of damage and modifications. Oxidative stress at the cellular level is reduced by enzymatic and nonenzymatic antioxidative mechanisms operating intracellularly [1,14]. Of these mechanisms, the heat shock (HS) response, involving the

Address correspondence to: Dr. Suresh I. S. Rattan, Department of Molecular and Structural Biology (IMSB), University of Aarhus, Gustav Wieds Vej 10-C, DK-8000 Aarhus -C, Denmark; Tel: +45 89 42 50 34; Fax: +45 86 12 31 78; E-Mail: rattan@imsb.au.dk.

HS family of stress proteins (HSP) and other enzymes, is activated under oxidative stress as well as other types of cellular and environmental challenges [15,16]. The HS response is a rapid cellular response against protein damages, since HSPs either protect proteins against denaturation and modifications by capping and refolding, or drive damaged proteins into appropriate proteolytic pathways [17–19]. It has been shown that repeated mild stresses, including HS, have beneficial effects on aging in cells and organisms [4,20]. Cellular response to mild stress is suggested to enhance its defense capacity to adapt to environmental changes and to survive in otherwise lethal conditions. Such a phenomenon is known as hormesis and has been widely observed in relation to irradiation, toxins, HS, and other stresses [20–22]. Hormetic pathways are still largely unknown but could act in delaying the breakdown of the cellular balance between pro- and antioxidative conditions and/or in maintaining HSP synthesis and activity. Our group has previously reported various antiaging hormetic effects of repeated mild HS (RMHS) on human fibroblasts in culture [4,23]. These effects included the maintenance of morphology and cytoskeletal organization during serial passaging in vitro. Since alterations in cellular morphology during serial passaging are accompanied by the accumulation of abnormal proteins [2,24], we have now investigated whether such preventive effects of RMHS on fibroblasts could be due to a decrease of protein oxidation and glycoxidation, and due to the maintenance of the redox status and/or the HSP production. Results presented in this report provide evidence in support of these possibilities.

MATERIALS AND METHODS

Cell culture and repeated mild heat shock

Primary cultures of normal diploid human skin fibroblasts (HSF) were established from skin biopsies obtained from healthy young female donors as described before [23]. Cells were grown in 75 cm^2 flasks (COSTAR, Cambridge, MA, USA) at 37°C, 5% CO_2, and 95% humidity, in DMEM medium (Biowhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (Biological Industries, Beit Haemek, Israel), 400 μM glutamine (Biowhittaker) and 100 U/ml penicillin/streptomycin (Biowhittaker). In the near-confluent state, cells were trypsinized and subcultured at 1:2 split ratio repeatedly until the end of their proliferative capacity in vitro, in accordance with the well-established protocols for the Hayflick system of replicative senescence [23, 25]. Cumulative population doubling level (CPDL) achieved at the end of replicative lifespan was designated as 100% lifespan completed. Parallel cultures were subjected to RMHS throughout their lifespan. HS was given by dipping the culture flasks for 1 h, twice a week, in a fine-regulated water bath set up at 41°C. Cultures were kept at 37°C for 30 min before the medium was changed. This protocol was followed with a condition that no HS was given to cells during the 24 h before and after subculturing [23]. This was to avoid any negative effects of HS on cell attachment after trypsinization. Cells were collected by trypsinization at different CPDL, washed twice in Hank's buffer and centrifuged (1000 $\times$ *g*, 5 min, 4°C). Cell pellet was collected and homogenized in ice-cold PBS (pH 7.4) containing a cocktail of protease inhibitors (aprotinin 5 μg/ml; leupeptin 5 μg/ml; antipain 5 μg/ml; PMSF 100 μg/ml; EDTA 1 mM). Cells were disrupted by sonication on ice (4 pulses of 30 s) and then centrifuged (16,000 $\times$ *g*, 30 min, 4°C). Protein content of the supernatant was determined using the Lowry method (Bio-Rad, Hercules, CA, USA). Supernatant was stored at -20°C.

Determination of HSP and oxidized carbonylated protein levels by Western blot analysis

Extraction of the cell samples used for the determination of HSP content was performed 48 h after the last HS. Briefly, 20 μg of protein from each extract was boiled for 5 min in Laemmli buffer and proteins were separated on a 10% or 12.5% SDS/PAGE, for HSP70 and HSP27, respectively. Proteins were electrically transferred to a nitrocellulose membrane (Hybond-C, Amersham, Piscataway, NJ, USA), and the membrane was blocked overnight at 4°C in PBS containing 5% nonfat dried milk. The homogeneity of protein transfer was controlled by a short Ponceau-red staining. Blots were washed with PBS containing 0.05% Tween 20 and then incubated for 90 min with either a mouse anti-HSP27 monoclonal Ig G antibody (1:1000, Stressgen, Victoria, BC, Canada) or a mouse anti-HSP70 monoclonal Ig G antibody (1:1500, Stressgen) diluted in PBS. After subsequent washes, blots were incubated for 1 h with HRP-conjugated goat polyclonal antimouse IgG antibody (1:1000, Dako, Glostrup, Denmark). After further washes, enhanced chemiluminescence (ECL) detection was performed according to the instructions of the manufacturer (Pierce, Rockford, IL, USA). Densitometric analysis of the spots on the autoradiography was performed using the Molecular Analyst 2.1.1 software (Bio-Rad).

Protein carbonyl content was measured by immunoblot detection using the Oxyblot protein oxidation detection kit (Intergen, Purchase, NY, USA), according to the manufacturer instructions. Briefly, 15 μg of protein from each cellular extract was incubated with dinitrophenylhydrazine (DNPH) for 15 min at room temperature.

Samples were neutralized, reduced by addition of 2-mercaptoethanol, and loaded into a 10% SDS-PAGE. After electroblotting, oxidized proteins were revealed by an anti-DNP antibody. Detection of DNP-proteins and quantification of spots on the autoradiographies were performed as described above.

Determination of Furosine level

Furosine formed during acid hydrolysis of the Amadori product was measured using HPLC chromatographic analysis. Briefly, 200 μg of protein were hydrolyzed in 2 ml of 6 N HCl for 24 h at 110°C in sealed glass ampoules (Wheaton, Millville, NJ, USA). Sample was dried, diluted in water and injected into a Peakmax C18 reverse-phase column (100 $\times$ 4.6 mm, 4 μm, Bio-Tek, Milano, Italy) and equilibrated with 0.5% acetic acid. Column was coupled to a HPLC system (522, Bio-Tek) and chromatographic analysis was carried out by isocratic elution with 0.5% acetic acid, at 0.6 ml/min flow rate. The effluent was monitored at 280 nm with a diode array detector (540+, Bio-Tek) and peaks were analyzed by the Kroma system 3000 software (Bio-Tek). Furosine eluted at ~2.5 min and was quantified according to a calibration curve using synthetic furosine diluted in 0.1 N HCl as reference (Neosystem, Strasbourg, France). Results were expressed as pmol furosine/μg protein.

Determination of total AGE products and CML-rich proteins levels

AGE assays were performed by competitive ELISA as previously described [26] except that 1,2-phenylenediamine-dihydrochloride substrate solution (OPD tablets) was used as peroxidase substrate. Absorbance was measured at dual wavelengths (490/655 nm) on a microplate reader (Bio-Rad). AGE content was quantified according to a calibration curve (0–20,000 A. U. AGE/ml).

Measurement of cellular CML-protein content was performed by direct ELISA. Protein concentrations of cytosolic extracts were adjusted at 500 ng/ml in carbonate buffer (Na_2CO_3 20 mM, $NaHCO_3$ 30 mM, pH 9.6) and triplicates consisting of 100 μl of each sample were coated (4°C, overnight) in 96 well microplates. After washes with PBS containing 0.05% Tween 20, samples were incubated (1 h, RT) with an anti-CML rabbit polyclonal IgG antibody (a gift from Dr. Toshio Miyata) diluted at 1:2000 in PBS. After extensive washes, wells were incubated (1 h, RT) with HRP-conjugated sheep polyclonal antirabbit IgG antibody (1:1000, Dako) and HRP was detected as described above.

Determination of cellular glutathione content

Cellular glutathione content was determined using a previously described kinetic assay with some modifications [27]. Cells were grown in 12 well culture plates. At subconfluence, the medium was sucked off and cells were washed twice with warm Hank's buffer. Cells were lysed by addition of 200 μl of 10 mM HCl to the wells and subjected to two short freezing/defreezing cycles on dry ice. The acid solution was transferred to a microcentrifuge tube and half of the samples were processed for oxidized glutathione (GSSG) determination. In that case, acid solution was incubated with 5 μl of 2-vinyl pyridine (Sigma-Aldrich, Steinheim, Germany) and 1 μl of 20% triethanolamine (Sigma-Aldrich) for 1 h to derivatize reduced glutathione (GSH). For the determination of total glutathione (GSH + GSSG) in the sample, this step was omitted. Samples were deproteinized by addition of 250 μl of sulfosalicylic acid (10% w/v) containing 4 mM EDTA. After 10 min of incubation on ice, the solution was centrifuged (12,500 $\times$ *g*, 5 min, 4°C). Pellet was resuspended in 300 μl of 0.4 M NaOH and the protein content was determined by the Lowry method (Bio-Rad). Supernatant was transferred into another tube and neutralized with 100 μl of K_2HPO_4.

For the determination of total glutathione and GSSG, 50 μl of the supernatant was added into a 96 well plate containing 100 μl of the following solution: 0.2 mM of 5,5′−dithiobis-2-nitrobenzoic acid; 0.3 mM of NADPH; 2U/ml of glutathione reductase diluted in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. Enzymatic reaction was carried out at 25°C. The initial rate of reaction was determined by increase of absorbance/min measured at 420/595 nm by using a microplate reader (Model 550, Bio-Rad) coupled to software dedicated to kinetic measurement (Microplate Manager, 5.0.1, Bio-Rad). Total glutathione and GSSG contents were determined in reference to standard curves (0–5 μM) of GSSG diluted in the same buffers. GSH concentration was calculated by subtracting the GSSG concentration from the total glutathione concentration in each sample. Results were expressed as nmol of glutathione/mg protein. The redox index, a measure of the oxidative stress of the cell, was determined by calculating the ratio of GSSG/GSH at different passage levels.

Statistical analysis

Data are expressed as means $\pm$ standard error of the mean (SEM) for all data. Effect of serial passaging and RMHS treatment on all parameters studied were tested for significance using analysis of variance (ANOVA) and Student's *t*-test; p values $< .05$ were considered to be statistically significant.

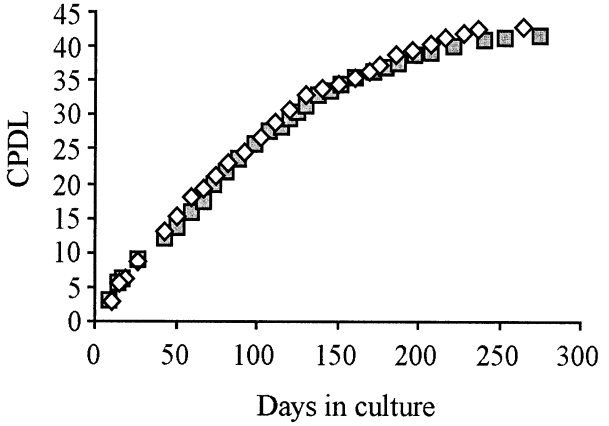

Fig. 1. Effect of RMHS on the growth and replicative lifespan of HSF in culture. Longevity curves of HSF show cumulative population doubling level (CPDL) achieved on serial passaging. Data are representative of two independent sets of cell cultures for control (◇) and RMHS (■) cells.

RESULTS

Cell growth and replicative senescence

Two independent cultures of HSF, which were comprised of at least 12 flasks of 75 cm^2 each and were serially passaged, achieved CPDL 40.1 ± 1.9 for the control cells and 40 ± 1.2 for the RMHS cells, over a 10 month period (Fig. 1). These CPDL are considered as 100% lifespan completed for the purposes of analysis of all data. During serial passaging, HSF exhibited progressive senescence as evidenced by changes in morphology, increased cell size, slower rate of population doubling, and increased number of neutral β-galactosidase-positive cells (data not shown). In agreement with a previously published report [23], in this series of experiments also, HSF could be exposed to more than 70 rounds of mild HS without any obvious harmful effects on cell attachment, growth rates, and other characteristics.

Effect of RMHS on accumulation of carbonylated proteins

The content of oxidized protein was determined by using the Oxyblot protein oxidation detection kit as described earlier. Carbonylated proteins accumulated during serial passaging in both groups of cells for up to 75% lifespan completed (Fig. 2). However, in late passage cells with 90% of the lifespan completed, RMHS protected low molecular weight (<45 kDa) and high molecular weight (>90 kDa) proteins from oxidation (Fig. 2A). Moreover, in late passage cells, the densitometric intensity (Fig. 2B) of spots of oxidized proteins were significantly lower in the RMHS group than in the control group. Two spots of proteins at about 60 and 72 kDa,

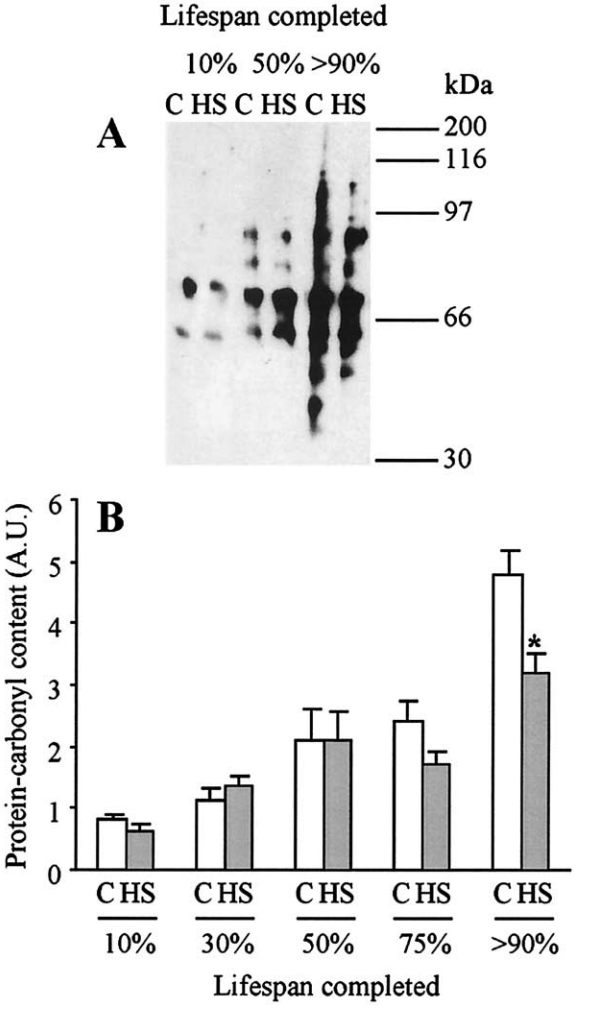

Fig. 2. Effect of RMHS on oxidized carbonylated protein content in HSF during serial passaging in vitro. (A) Immunochemical detection of carbonylated proteins. (B) Densitometric analysis of the autoradiographs. Data are the result of five to seven independent determinations performed on individual samples. C = control cells; HS = RMHS cells. *Significantly different from age-matched control ($p < .05$).

were observed to be oxidized even in the early passage young cells. Identities of these and other oxidized proteins have not been established in this study.

Effect of RMHS on accumulation of glycation products

Presence of early glycation products or Amadori products (AP) was determined by measuring the furosine concentration resulting from the acid hydrolysis of AP. Furosine content increased 3-fold during serial passaging, from 0.53 ± 0.21 pmol/μg protein in young early passage control cells (less than 25% lifespan completed) to 1.6 ± 0.42 pmol/μg protein in late passage control cells (more than 85% lifespan completed; Fig. 3). The levels of furosine also increased in RMHS cells during

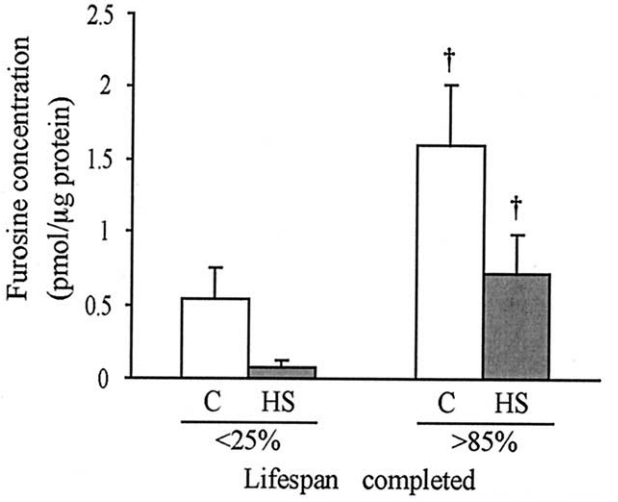

Fig. 3. Effect of RMHS on the furosine content in HSF during serial passaging in vitro. Data are the result of three to seven independent determinations performed on individual samples. C = control cells; HS = RMHS cells. †Significantly different from young-matched HSF ($p < .05$).

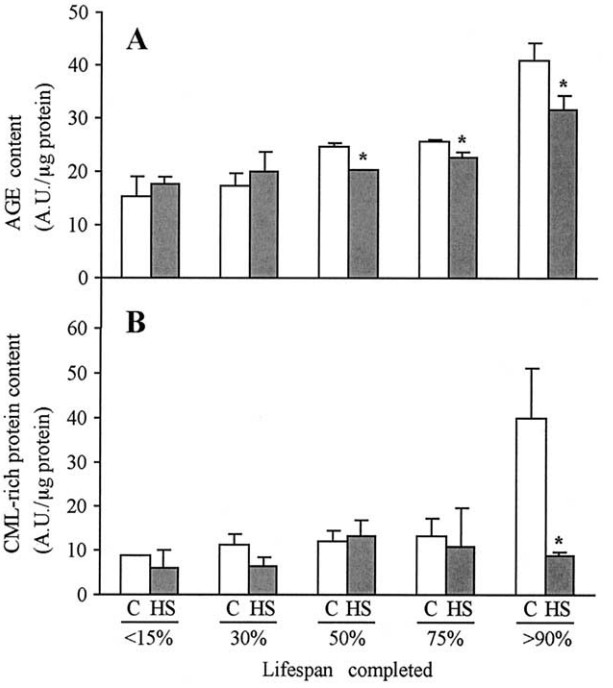

Fig. 4. Effect of RMHS on AGE/glycoxidized content (A) and on the CML-rich protein content (B) in HSF during serial passaging in vitro. Data are the result of 4–10 independent determinations performed on individual samples, with triplicates in each determination. C = control cells; HS = RMHS cells. *Significantly different from age-matched control ($p < .05$).

serial passaging, from 0.08 ± 0.04 pmol/μg in early passage cells to 0.72 ± 0.27 pmol/μg in late passage cells. RMHS reduced the concentration of furosine in both early passage and late passage cells, but the effects were not statistically significant.

Total AGE/glycoxidation products and CML-rich protein concentrations were measured by ELISA. AGE/glycoxidation products concentration increased during serial passaging in control and RMHS cells (Fig. 4A). However, after about 50% lifespan completed, the accumulation of AGE proteins was significantly slower in RMHS cells than in control cells. In comparison, CML-rich protein concentration was steady up to 75% of the lifespan of HSF and increased 3-fold over 90% of the lifespan completed in the control but not in the RMHS group (Fig. 4B).

Effect of RMHS on the basal glutathione concentration

To investigate whether a decrease in the accumulation of oxidatively damaged proteins was due to a lower oxidative stress in HSF subjected to RMHS, we have measured the concentration of reduced (GSH) and oxidized (GSSG) glutathione during serial passaging. In early passage young cells, the basal concentration of GSH in the RMHS cells (115.2 ± 5.1 nmol/mg protein) was 2.5 times higher than that in control cells (45.9 ± 4.6 nmol/mg protein). In the case of GSSG, small quantities (4.9 ± 1.6 nmol/mg protein) were detectable in early passage young control cells only. As an index of the cellular oxidative stress, the ratio GSSG/GSH was much lower in early passage RMHS cells *(not detectable)* than that in late passage control fibroblasts (0.11 ± 0.03). During cellular replicative senescence, the basal GSH concentration decreased by 33% and 52% both in control and RMHS cells, respectively (Fig. 5). However, even in late passage cells, the basal concentration of GSH was significantly higher (56 ± 4.1 nmol/mg protein) and the GSSG concentration was significantly lower (3 ± 0.1 nmol/mg protein) in the RMHS cells than that in the control cells (7.1 ± 0.3 nmol/mg protein). Moreover, the GSSG/GSH ratio was more than 4 times higher in late passage control cells (0.23 ± 0.003) than in late passage RMHS cells (0.05 ± 0.004).

Effect of RMHS on oxidative-related HSP level

Since HSP27 is known to be involved in the maintenance of the pool of GSH in murine cell lines [28–30], we have determined the basal levels of HSP27 in HSF during serial passaging. Western blot analysis of early passage young HSF and late passage senescent HSF showed that the levels of HSP27 was 2.5–3 times more in young cells than in senescent cells (Fig. 6A). However, HSP27 levels were identical in control and RMHS cells both in early passage and in late passage cells. We have also measured the basal levels of HSP70, another mo-

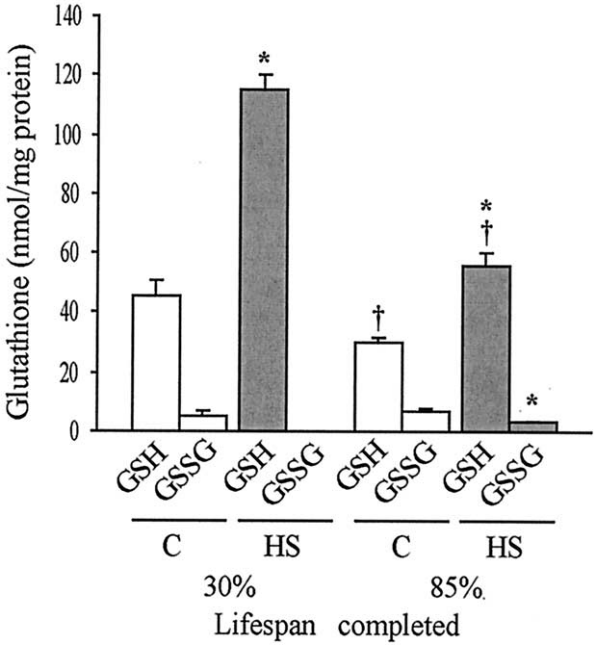

Fig. 5. Effect of RMHS on the basal concentration of GSH and GSSG in HSF during serial passaging in vitro. RMHS cells have received one mild HS 48 h before the cell extraction. Data are the result of 10–15 independent determinations performed on individual samples, with duplicates in each determination. C = control cells; HS = RMHS cells. *Significantly different from age-matched control ($p < .05$), †significantly different from young-matched HSF ($p < .05$).

lecular chaperone involved in the cellular response to oxidative stress. HSP70 in early passage control HSF was undetectable, but this inducible chaperone was present at detectable levels in young RMHS cells 48 h after the last mild HS (Fig. 6B). During serial passaging, the basal expression of HSP70 increased in both groups of cells and its level was two times higher in the late passage RMHS cells than that in the control cells.

DISCUSSION

The present study reports further biochemical analysis of the beneficial effects of repeated exposure of human skin fibroblasts to mild heat stress. Since accumulation of oxidative and glycoxidative modified abnormal proteins is a hallmark of cellular replicative senescence in vitro [1–5,31,32], we have shown that RMHS limits the accumulation of such modified proteins during serial passaging of human skin fibroblasts.

Protein oxidation and glycoxidation are not random processes and specific target proteins as well as specific sites on these proteins are susceptible to such modifications during aging [2,9,33–36]. Increase of oxidation/glycoxidation processes with age may reflect the imbalance between ROS production and the rate at which damaged proteins are repaired or degraded. Presence of furosine in cultured fibroblasts reflects the presence of Amadori product (AP) and shows that the Maillard pathway can also occur intracellularly in mitotic cells. The formation of AP is selectively due to the nonoxidative reaction of sugars with the free amine of a lysine in a protein [8]. We have observed a slight but significant accumulation of AP in HSF during serial passaging, suggesting that either this pathway is accelerated or that the turnover of AP is decreased during replicative senescence. Activation of the Maillard pathway during replicative senescence could be also due to misregulation of glycolysis, shifting glucose catabolism into parallel path-

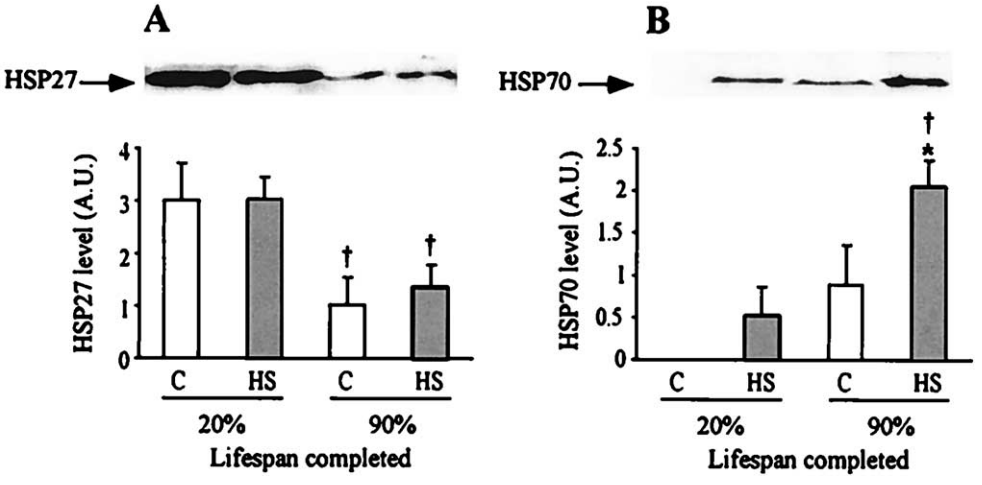

Fig. 6. Effect of RMHS on the basal level of HSP27 (A) and HSP70 (B) in HSF during serial passaging in vitro. Top: typical immunochemical detection of HSP27 and HSP70. Bottom: densitometric analysis of the autoradiographs. Data are the result of six independent determinations performed on individual samples. C = control cells; HS = RMHS cells. *Significantly different from age-matched control ($p < .05$), †significantly different from young-matched HSF ($p < .05$).

ways [37]. In addition, increase in the AP concentration during replicative senescence may be due to an attenuation of potential mechanisms of repair and/or degradation of these molecules. We have also shown a progressive accumulation of intracellular AGE/glycoxidation products during serial passaging, which might be due to a higher concentration of metabolism fragmentation products such as α-oxoaldehydes, or by an acceleration of the AP remodelling [38].

In contrast to the progressive accumulation of AGE/glycoxidation products during serial passaging of HSF, increase in CML levels was observed in late passage cultures only after more than 90% lifespan had been completed. This indicates that the glycoxidation pathway is well regulated during the first part of HSF lifespan. However, CML has multiple sources, including sugars, serine myeloperoxidase, lipid peroxidation and ascorbate oxidation reactions [39], and a misregulation of redox balance could lead to increased level of ROS catalyzing the formation of glycoxidation products and CML. Such an imbalance of the cellular redox potential can also be one of the reasons for the accumulation of carbonylated proteins observed in the present study and during aging as reported previously [1,2]. Accumulation of intracellular carbonyls and AGE/glycoxidation products such as pentosidine, vesperlysine, crossline, carboxyethyl-lysine, and CML have been reported to occur in several cell types with age, and some of those damages have been considered as reliable markers of aging and longevity [40–42].

Our results show that RMHS can limit the accumulation of oxidative/glycoxidative markers during serial passaging of HSF. These results led us to investigate whether the effect of RMHS could be due to the maintenance of the redox potential in the cytosol of fibroblasts leading to a higher resistance to oxidative stress during aging. The intracellular pool of the tripeptide glutathione, estimated to contain more than 90% of all cellular reducing equivalents, is of crucial importance for resistance to oxidative stress [43]. We have shown that the basal concentration of reduced glutathione (GSH) is much higher and the level of oxidized glutathione is lower in RMHS cells than in control cells throughout the HSF lifespan in vitro. However, GSH concentration drops in both groups of cells during serial passaging. The redox index of the cell, measured by the ratio GSSG/GSH, increased in both groups of cells during serial passaging, and was much higher in control cells than in RMHS cells throughout the lifespan. Similar results have been reported previously for fibroblasts and for other vertebrate and invertebrate models [44–48].

In a separate series of experiments, we have observed that senescent HSF have a reduced ability to respond to oxidative stress by maintaining GSH level as compared with RMHS cells (unpublished observation). Thus, we can postulate that RMHS allows HSF to resist oxidation by either helping the GSH redox cycle or by activating the synthesis of GSH, which is known to be downregulated during aging [49,50]. Previous studies have reported that mild heat stress to mammalian cells and exercise stress in rats increased the level of GSH in cells and tissues [51,52]. This could be due to an increase of expression of γ-glutamyl-cysteine synthetase and GSH synthetase, whose genes are regulated by a heat shock promoter [53]. The lower concentration of GSH in control fibroblasts may contribute to the accumulation of damaged proteins, since it has been shown that some posttranslational modifications of proteins can take place only when GSH is depleted [54]. Moreover, the glyoxalase pathway, detoxifying precursors of glycoxidation products, is GSH-dependent and one could attribute the loss of activity of this pathway during aging to a lower GSH concentration [55,56]. That could partly explain the observed accumulation of glycoxidation products such as CML during serial passaging in vitro.

GSH regulation is also tightly linked to the heat shock response, the activation of the heat shock transcription factors, and the induction of HSP such as α-crystalline, HSP27, and HSP70 [57–59]. HSP27 and HSP70 interact with specific or nonnative proteins to cap or to refold them during and after stress. Large unphosphorylated aggregates of HSP27 help the GSH redox cycle by increasing the pool of GSH in cytosol [28–30]. This pro-GSH effect of HSP27 is in agreement with the maintenance of the GSH pool in RMHS cells observed in our study. Since HSP27 content was identical in both groups of cells and decreased during serial passaging, that leads us to conclude that the maintenance of the GSH concentration in RMHS cells is not due to an overexpression of HSP27. Such age-related expression profile of HSP27 has been previously reported for *Drosophila* [60,61]. It is also possible that the hormetic effects of RMHS favor the preferential accumulation of an active unphosphorylated aggregated form of HSP27. In comparison, the basal level of HSP70, which is a major inducible system involved in defense against stress, increases during serial passaging in both control and RMHS senescent cells. Age-related increase of HSP70 has been described for rats and *Drosophila* and is suggested to reflect the accumulation of denatured and damaged protein initiating the HS response [61–64]. Pretreatment of HSF by RMHS may initiate de novo production of HSP70 during serial passaging and may protect intracellular proteins more efficiently against stress. Previous studies have demonstrated that mild-stress pretreatment induces the accumulation of HSP70 and reduces cellular damage [65,66] and that overexpression of HSP70 in transgenic models is

associated with a decrease of protein aggregation [67–69].

Finally, this is the first report that shows the beneficial hormetic effects of RMHS on the redox status and the level of damaged proteins in human cells during serial passaging. It is clear that through hormesis, the activity and efficiency of various defense mechanisms can be improved and/or maintained even in senescent cells, leading to limitation of oxidative and glycoxidative damages. Nevertheless, oxidized and glycoxidized protein accumulation is probably not the ultimate cause of replicative senescence in HSF because limitation of these products by RMHS does not lead to the prolongation of the proliferative lifespan of cells. However, a decreased accumulation of such harmful products by repeated mild stress helps in maintaining the cell structure and function during the limited lifespan of cells in culture. These results further strengthen the possibility of applying hormesis in aging research and therapy [70].

Acknowledgements — We thank Gunhild Siboska and Helle Jakobsen for expert technical assistance, and Jesper S. Pedersen for his expertise in the preparation of the figures. We are grateful to Dr. T. Miyata (Tokai University, Japan) and Dr. H. Bakala (Paris 7 University, France) for the generous gift of anti-CML and anti-AGE antibodies, respectively. This study was supported by funds from the shared cost action program GENAGE and PROTAGE under the EU Biomed & Health Program Projects.

REFERENCES

[1] Finkel, T.; Holbrook, N. J. Oxidants, oxidative stress and the biology of ageing. *Nature* **408:**239–247; 2000.
[2] Berlett, B. S.; Stadtman, E. R. Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **272:**20313–20316; 1997.
[3] Baynes, J. W. From life to death. The struggle between chemistry and biology during aging. The Maillard reaction as an amplifier of genomic damage. *Biogerontology* **1:**235–246; 2000.
[4] Verbeke, P.; Clark, B. F. C.; Rattan, S. I. S. Modulating cellular aging in vitro: hormetic effects of repeated mild heat stress on protein oxidation and glycation. *Exp. Gerontol.* **35:**787–794; 2000.
[5] Rattan, S. I. S. Synthesis, modifications, and turnover of proteins during aging. *Exp. Gerontol.* **31:**33–47; 1996.
[6] Davies, M. J.; Fu, S.; Wang, H.; Dean, R. T. Stable markers of oxidant damage to proteins and their application in the study of human disease. *Free Radic. Biol. Med.* **27:**1151–1163; 1999.
[7] Singh, R.; Barden, A.; Mori, T.; Beilin, L. Advanced glycation end-products: a review. *Diabetologia* **44:**129–146; 2001.
[8] Baynes, J. W.; Watkins, N. G.; Fisher, C. I.; Hull, C. J.; Patrick, J. S.; Ahmed, M. U.; Dunn, J. A.; Thorpe, S. R. The Amadori product on protein: structure and reactions. *Prog. Clin. Biol. Res.* **304:**43–67; 1989.
[9] Dukic-Stefanovic, S.; Schinzel, R.; Riederer, P.; Münch, G. AGES in brain ageing: AGE-inhibitors as neuroprotective and anti-dementia drugs? *Biogerontology* **2:**19–34; 2001.
[10] Miyata, T.; Kurokawa, K.; Van Ypersele De Strihou, C. Advanced glycation and lipoxidation end products: role of reactive carbonyl compounds generated during carbohydrate and lipid metabolism. *J. Am. Soc. Nephrol.* **11:**1744–1752; 2000.
[11] Baynes, J. W.; Thorpe, S. R. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes* **48:**1–9; 1999.
[12] Monnier, V. M.; Nagaraj, R. H.; Portero-Otin, M.; Glomb, M.; Elgawish, A. H.; Sell, D. R.; Friedlander, M. A. Structure of advanced Maillard reaction products and their pathological role. *Nephrol. Dial. Transplant.* **11:**20–26; 1996.
[13] Miyata, T.; Maeda, K.; Kurokawa, K.; Van Ypersele De Strihou, C. Oxidation conspires with glycation to generate noxious advanced glycation end products in renal failure. *Nephrol. Dial. Transplant.* **12:**255–258; 1997.
[14] Halliwell, B. Antioxidant defence mechanisms: from the beginning to the end (of the beginning). *Free Radic. Res.* **31:**261–272; 1999.
[15] Freeman, M. L.; Borrelli, M. J.; Meredith, M. J.; Lepock, J. R. On the path to the heat shock response: destabilization and formation of partially folded protein intermediates, a consequence of protein thiol modification. *Free Radic. Biol. Med.* **26:**737–745; 1999.
[16] Morimoto, R. I. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.* **12:** 3788–3796; 1998.
[17] Morimoto, R. I.; Kline, M. P.; Bimston, D. N.; Cotto, J. J. The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. *Essays Biochem.* **32:**17–29; 1997.
[18] Landry, J.; Huot, J. Modulation of actin dynamics during stress and physiological stimulation by a signaling pathway involving p38 MAP kinase and heat-shock protein 27. *Biochem. Cell Biol.* **73:**703–707; 1995.
[19] Mathew, A.; Morimoto, R. I. Role of the heat-shock response in the life and death of proteins. *Ann. N. Y. Acad. Sci.* **851:**99–111; 1998.
[20] Minois, N. Longevity and aging: beneficial effects of exposure to mild stress. *Biogerontology* **1:**15–29; 2000.
[21] Calabrese, E. J.; Baldwin, L. A. Hormesis: U-shaped dose responses and their centrality in toxicology. *Trends Pharmacol. Sci.* **22:**285–291; 2001.
[22] Rattan, S. I. S. Biogerontology: the next step. *Ann. N. Y. Acad. Sci.* **908:**282–290; 2000.
[23] Rattan, S. I. S. Repeated mild heat shock delays ageing in cultured human skin fibroblasts. *Biochem. Mol. Biol. Int.* **45:**753–759; 1998.
[24] Rattan, S. I. S. Ageing—a biological perspective. *Mol. Aspects Med.* **16:**439–508; 1995.
[25] Cristofalo, V. J.; Charpentier, R.; Phillips, P. D.; Celis, R. E. Serial propagation of human fibroblasts. In: Celis, J. E., ed. *Cell biology: a laboratory handbook.* San Diego: Academic Press; 1998:321–326.
[26] Teillet, L.; Verbeke, P.; Gouraud, S.; Bakala, H.; Borot-Laloi, C.; Heudes, D.; Bruneval, P.; Corman, B. Food restriction prevents advanced glycation end product accumulation and retards kidney aging in lean rats [In Process Citation]. *J. Am. Soc. Nephrol.* **11:**1488–1497; 2000.
[27] Almazan, G.; Liu, H. N.; Khorchid, A.; Sundararajan, S.; Martinez-Bermudez, A. K.; Chemtob, S. Exposure of developing oligodendrocytes to cadmium causes HSP72 induction, free radical generation, reduction in glutathione levels, and cell death. *Free Radic. Biol. Med.* **29:**858–869; 2000.
[28] Baek, S. H.; Min, J. N.; Park, E. M.; Han, M. Y.; Lee, Y. S.; Lee, Y. J.; Park, Y. M. Role of small heat shock protein HSP25 in radioresistance and glutathione-redox cycle. *J. Cell. Physiol.* **183:** 100–107; 2000.
[29] Mehlen, P.; Hickey, E.; Weber, L. A.; Arrigo, A. P. Large unphosphorylated aggregates as the active form of hsp27 which controls intracellular reactive oxygen species and glutathione levels and generates a protection against TNFalpha in NIH-3T3-ras cells. *Biochem. Biophys. Res. Commun.* **241:**187–192; 1997.
[30] Mehlen, P.; Kretz-Remy, C.; Preville, X.; Arrigo, A. P. Human hsp27, Drosophila hsp27 and human alphaB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNFalpha-induced cell death. *EMBO J.* **15:**2695–2706; 1996.
[31] Thorpe, S. R.; Baynes, J. W. Role of the Maillard reaction in

diabetes mellitus and diseases of aging. *Drugs Aging* **9:**69–77; 1996.

[32] Lee, A. T.; Cerami, A. Role of glycation in aging. *Ann. N. Y. Acad. Sci.* **663:**63–70; 1992.

[33] Yan, L. J.; Levine, R. L.; Sohal, R. S. Oxidative damage during aging targets mitochondrial aconitase. *Proc. Natl. Acad. Sci. USA* **94:**11168–11172; 1997.

[34] Requena, J. R.; Stadtman, E. R. Conversion of lysine to N(epsilon)-(carboxymethyl)lysine increases susceptibility of proteins to metal-catalyzed oxidation. *Biochem. Biophys. Res. Commun.* **264:** 207–211; 1999.

[35] Munch, G.; Schinzel, R.; Loske, C.; Wong, A.; Durany, N.; Li, J. J.; Vlassara, H.; Smith, M. A.; Perry, G.; Riederer, P. Alzheimer's disease—synergistic effects of glucose deficit, oxidative stress and advanced glycation endproducts. *J. Neural Transm.* **105:**439–461; 1998.

[36] Dukan, S.; Farewell, A.; Ballesteros, M.; Taddei, F.; Radman, M.; Nystrom, T. Protein oxidation in response to increased transcriptional or translational errors. *Proc. Natl. Acad. Sci. USA* **97:** 5746–5749; 2000.

[37] Pastoris, O.; Boschi, F.; Verri, M.; Baiardi, P.; Felzani, G.; Vecchiet, J.; Dossena, M.; Catapano, M. The effects of aging on enzyme activities and metabolite concentrations in skeletal muscle from sedentary male and female subjects. *Exp. Gerontol.* **35:**95–104; 2000.

[38] Abordo, E. A.; Minhas, H. S.; Thornalley, P. J. Accumulation of alpha-oxoaldehydes during oxidative stress: a role in cytotoxicity. *Biochem. Pharmacol.* **58:**641–648; 1999.

[39] Fu, M. X.; Requena, J. R.; Jenkins, A. J.; Lyons, T. J.; Baynes, J. W.; Thorpe, S. R. The advanced glycation end product, Nε-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J. Biol. Chem.* **271:**9982–9986; 1996.

[40] Agarwal, S.; Sohal, R. S. Relationship between susceptibility to protein oxidation, aging, and maximum life span potential of different species. *Exp. Gerontol.* **31:**365–372; 1996.

[41] Sell, D. R.; Lane, M. A.; Johnson, W. A.; Masoro, E. J.; Mock, O. B.; Reiser, K. M.; Fogarty, J. F.; Cutler, R. G.; Ingram, D. K.; Roth, G. S.; Monnier, V. M. Longevity and the genetic determination of collagen glycoxidation kinetics in mammalian senescence. *Proc. Natl. Acad. Sci. USA* **93:**485–490; 1996.

[42] Sell, D. R.; Kleinman, N. R.; Monnier, V. M. Longitudinal determination of skin collagen glycation and glycoxidation rates predicts early death in C57BL/6NNIA mice. *FASEB J.* **14:**145–156; 2000.

[43] Hayes, J. D.; McLellan, L. I. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radic. Res.* **31:**273–300; 1999.

[44] Allen, R. G.; Tresini, M.; Keogh, B. P.; Doggett, D. L.; Cristofalo, V. J. Differences in electron transport potential, antioxidant defenses, and oxidant generation in young and senescent fetal lung fibroblasts (WI-38). *J. Cell. Physiol.* **180:**114–122; 1999.

[45] Sanz, N.; Diez-Fernandez, C.; Alvarez, A.; Cascales, M. Age-dependent modifications in rat hepatocyte antioxidant defense systems. *J. Hepatol.* **27:**525–534; 1997.

[46] Sohal, R. S.; Arnold, L.; Orr, W. C. Effect of age on superoxide dismutase, catalase, glutathione reductase, inorganic peroxides, TBA-reactive material, GSH/GSSG, NADPH/NADP+ and NADH/NAD+ in Drosophila melanogaster. *Mech. Ageing Dev.* **56:**223–235; 1990.

[47] Rathbun, W. B.; Murray, D. L. Age-related cysteine uptake as rate-limiting in glutathione synthesis and glutathione half-life in the cultured human lens. *Exp. Eye Res.* **53:**205–212; 1991.

[48] Nakata, K.; Kawase, M.; Ogino, S.; Kinoshita, C.; Murata, H.; Sakaue, T.; Ogata, K.; Ohmori, S. Effects of age on levels of cysteine, glutathione and related enzyme activities in livers of mice and rats and an attempt to replenish hepatic glutathione level of mouse with cysteine derivatives. *Mech. Ageing Dev.* **90:**195–207; 1996.

[49] Liu, R.; Choi, J. Age-associated decline in gamma-glutamylcysteine synthetase gene expression in rats. *Free Radic. Biol. Med.* **28:**566–574; 2000.

[50] Rathbun, W. B.; Holleschau, A. M. The effects of age on glutathione synthesis enzymes in lenses of Old World simians and prosimians. *Curr. Eye Res.* **11:**601–607; 1992.

[51] Ohkuwa, T.; Sato, Y.; Naoi, M. Glutathione status and reactive oxygen generation in tissues of young and old exercised rats. *Acta Physiol. Scand.* **159:**237–244; 1997.

[52] Will, O.; Mahler, H. C.; Arrigo, A. P.; Epe, B. Influence of glutathione levels and heat-shock on the steady-state levels of oxidative DNA base modifications in mammalian cells. *Carcinogenesis* **20:**333–337; 1999.

[53] Sugiyama, K.; Izawa, S.; Inoue, Y. The Yap1p-dependent induction of glutathione synthesis in heat shock response of Saccharomyces cerevisiae. *J. Biol. Chem.* **275:**15535–15540; 2000.

[54] Aquilina, J. A.; Carver, J. A.; Truscott, R. J. Oxidation products of 3-hydroxykynurenine bind to lens proteins: relevance for nuclear cataract. *Exp. Eye Res.* **64:**727–735; 1997.

[55] McLellan, A. C.; Thornalley, P. J. Glyoxalase activity in human red blood cells fractioned by age. *Mech. Ageing Dev.* **48:**63–71; 1989.

[56] Sharma-Luthra, R.; Kale, R. K. Age related changes in the activity of the glyoxalase system. *Mech. Ageing Dev.* **73:**39–45; 1994.

[57] Calabrese, V.; Testa, G.; Ravagna, A.; Bates, T. E.; Stella, A. M. HSP70 induction in the brain following ethanol administration in the rat: regulation by glutathione redox state. *Biochem. Biophys. Res. Commun.* **269:**397–400; 2000.

[58] Zou, J.; Salminen, W. F.; Roberts, S. M.; Voellmy, R. Correlation between glutathione oxidation and trimerization of heat shock factor 1, an early step in stress induction of the Hsp response. *Cell Stress Chaperones* **3:**130–141; 1998.

[59] Liu, H.; Lightfoot, R.; Stevens, J. L. Activation of heat shock factor by alkylating agents is triggered by glutathione depletion and oxidation of protein thiols. *J. Biol. Chem.* **271:**4805–4812; 1996.

[60] King, V.; Tower, J. Aging-specific expression of Drosophila hsp22. *Dev. Biol.* **207:**107–118; 1999.

[61] Wheeler, J. C.; Bieschke, E. T.; Tower, J. Muscle-specific expression of Drosophila hsp70 in response to aging and oxidative stress. *Proc. Natl. Acad. Sci. USA* **92:**10408–10412; 1995.

[62] Chin, J. H.; Okazaki, M.; Hu, Z. W.; Miller, J. W.; Hoffman, B. B. Activation of heat shock protein (hsp)70 and proto-oncogene expression by alpha1 adrenergic agonist in rat aorta with age. *J. Clin. Invest.* **97:**2316–2323; 1996.

[63] Maiello, M.; Boeri, D.; Sampietro, L.; Pronzato, M. A.; Odetti, P.; Marinari, U. M. Basal synthesis of heat shock protein 70 increases with age in rat kidneys. *Gerontology* **44:**15–20; 1998.

[64] Locke, M. Heat shock transcription factor activation and hsp72 accumulation in aged skeletal muscle. *Cell Stress Chaperones* **5:**45–51; 2000.

[65] Baek, S. H.; Kim, J. Y.; Choi, J. H.; Park, E. M.; Han, M. Y.; Kim, C. H.; Ahn, Y. S.; Park, Y. M. Reduced glutathione oxidation ratio and 8 ohdG accumulation by mild ischemic pretreatment. *Brain Res.* **856:**28–36; 2000.

[66] Amin, V.; Cumming, D. V.; Latchman, D. S. Over-expression of heat shock protein 70 protects neuronal cells against both thermal and ischaemic stress but with different efficiencies. *Neurosci Lett.* **206:**45–48; 1996.

[67] Krobitsch, S.; Lindquist, S. Aggregation of huntingtin in yeast varies with the length of the polyglutamine expansion and the expression of chaperone proteins. *Proc. Natl. Acad. Sci. USA* **97:**1589–1594; 2000.

[68] Plumier, J. C.; Krueger, A. M.; Currie, R. W.; Kontoyiannis, D.; Kollias, G.; Pagoulatos, G. N. Transgenic mice expressing the human inducible Hsp70 have hippocampal neurons resistant to ischemic injury. *Cell Stress Chaperones* **2:**162–167; 1997.

[69] Tatar, M.; Khazaeli, A. A.; Curtsinger, J. W. Chaperoning extended life. *Nature* **390:**30; 1997.

[70] Rattan, S. I. S. Applying hormesis in aging research and therapy. *Hum. Exp. Toxicol.* **20:**281–285; 2001.

ABBREVIATIONS

AGE—advanced glycation endproduct
AP—Amadori product
CML—Nε−carboxymethyl-lysine
CPDL—cumulative population doubling level
DNPH—2,4-dinitrophenylhydrazine
ELISA—enzyme-linked immunosorbent assay
GSH—reduced glutathione
GSSG—oxidized glutathione
HPLC—high performance liquid chromatography
HS—heat shock
HSF—human skin fibroblast
HSP—heat shock protein
PAGE—polyacrylamide gel electrophoresis
RMHS—repeated mild heat shock
ROS—radical oxygen species