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Experimental Gerontology 37 (2002) 1223–1228

Experimental Gerontology

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Mild stress-induced stimulation of heat-shock protein synthesis and improved functional ability of human fibroblasts undergoing aging in vitro

Jannik Fonager, Rasmus Beedholm, Brian F.C. Clark, Suresh I.S. Rattan*

Laboratory of Cellular Ageing, Department of Molecular Biology, Danish Centre for Molecular Gerontology, University of Aarhus, Gustav Wieds Vej 10-C, DK-8000 Aarhus-C, Denmark

Received 18 May 2002; received in revised form 4 June 2002; accepted 5 June 2002

Abstract

Repeated mild heat-shock (RMHS) treatment has anti-aging hormetic effects on human fibroblasts undergoing aging in vitro. Since heat and various other stresses induce the transcription and translation of heat-shock proteins (Hsp), it was investigated if RMHS treatment affected the basal levels of four major stress proteins Hsp27, 70, 90 and Hsc70. The basal levels of Hsp27, Hsc70, and Hsp70 increased significantly in late passage senescent cells, which is indicative of an adaptive response to cumulative intracellular stress during aging. RMHS increased the levels of these Hsp even in early passage young cells and were maintained high throughout their replicative lifespan. In comparison, the amount of Hsp90 decreased both with aging and RMHS treatment in vitro. However, whereas the difference in the levels of Hsp70 and Hsp90 was statistically significant, the levels of Hsp27 and Hsc70 were statistically similar in normal and RMHS-treated serially passaged cells. These alterations were accompanied by an improved functional and survival ability of the cells in terms of increased proteasomal activities, increased ability to decompose H_2O_2, reduced accumulation of lipofuscin and enhanced resistance to ethanol, H_2O_2 and UV-A radiation.

Keywords: Anti-aging; Stress; Heat shock; Hormesis; Proteasome; Catalase

1. Introduction

We have previously reported that repeated mild heat-shock (RMHS) treatment had anti-aging effects on human skin fibroblasts during serial passaging in culture. These effects included reduced cell enlargement, reduced accumulation of oxidized and glycoxidized abnormal proteins, increased ratio of reduced and oxidized glutathione, and increased resistance to sugar-induced damage to proteins (Rattan, 1998; Verbeke et al., 2001a, 2002). Such a phenomenon where biologically beneficial effects of mild stress are observed as a result of either over compensatory adaptive response or direct stimulatory response is termed hormesis, and its application in biogerontological research and therapy is being explored (Minois, 2000; Calabrese and Baldwin, 2001; Rattan, 2001). Since we have

* Corresponding author. Tel.: +45-8942-5034; fax: +45-8612-3178.

E-mail address: rattan@imsb.au.dk (S.I.S. Rattan).

0531-5565/02/$ - see front matter
PII: S0531-5565(02)00128-6

used RMHS as a hormetic agent, it is important to find out what happens to various components of the heat-shock (HS) response during such treatments.

HS response is one of the primordial intracellular defense mechanisms against stressful conditions, which leads to the preferential transcription and translation of heat-shock proteins (Hsp). Optimal HS response in terms of Hsp synthesis and activity is essential for cell survival, whereas inefficient and altered HS response has been implicated in abnormal growth, development, aging and apoptosis (Söti and Csermely, 2000; Verbeke et al., 2001b). Genes encoding Hsp are highly conserved, and many of their products can be assigned to families on the basis of sequence homology and molecular weight. In mammals, various Hsp families comprise multiple members that differ in inducibility, intracellular localization and function. Hsp are known to play diverse roles as chaperones and/or as proteases. In unstressed cells, Hsp act in successful folding, assembly, intracellular localization, secretion, regulation and degradation of other proteins. Under conditions in which protein folding is perturbed or proteins begin to unfold and denature, Hsp assist in protein refolding, in protecting cellular systems against protein damages, in solubilizing aggregates to some extent, in sequestering overloaded and damaged proteins into larger aggregates and in targeting damaged proteins to degradation machinery (Verbeke et al., 2001b).

In this study, the effect of RMHS treatment on protein synthesis profiles of four major Hsp of human skin fibroblasts were investigated together with an analysis of other functional characteristics, such as proteasome activities, H_2O_2 decomposing ability, and survival after exposure to various stresses. Our results show that RMHS alters the Hsp profile accompanied by enhanced stress resistance and elevated proteasome activities.

2. Materials and methods

2.1. Cell culture, RMHS and sample collection

Primary cultures of normal diploid human skin fibroblasts, designated ASF-1, were established from mammary skin biopsies obtained from a healthy young female donor as described earlier (Rattan, 1998; Verbeke et al., 2001a). Cells were grown in 75 cm^2 plastic flasks (COSTAR, Cambridge, MA) at 37 °C, 5% CO_2, and 95% humidity, in DMEM (Biowhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (Biological Industries, Beit Haemek, Israel), 400 μM glutamine and 100 U/ml penicillin/streptomycin (Biowhittaker). In the near-confluent state, cells were trypsinized and subcultured at a 1:2 split ratio (while keeping a record of the input and output of number of cells) repeatedly until the end of their proliferative capacity in vitro and an account of cumulative population doubling level (CPDL) achieved was kept in accordance with the well-established protocols for the Hayflick system of replicative senescence (Rattan, 1998; Verbeke et al., 2001a). In this series of experiments CPDL achieved at the end of replicative lifespan of ASF-1 cells was 39, which was designated as 100% lifespan completed. Parallel cultures were subjected to RMHS throughout their lifespan 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. Cells were collected by trypsinization at different CPDL, washed twice in Hank's buffer and centrifuged (1000*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*g*, 30 min, 4 °C). Protein content of the supernatant was determined using the Lowry method (Bio-Rad, Hercules, CA). Supernatant was stored at − 20 °C.

2.2. Protein extraction and Western blotting

Extraction of the cell samples used for the determination of Hsp content was performed 72 h after the last HS. Briefly, 30 μg of protein from each extract was boiled for 5 min in loading buffer and proteins were separated on a 12.5% SDS/PAGE. Proteins were electrically transferred to a nitrocellulose membrane (Hybond-C Amersham, Piscataway, NJ), and the membrane was blocked overnight at 4 °C

in PBS containing 5% non-fat dried milk. Blots were washed with PBS containing 0.05% Tween 20 and then incubated for 60 min with one of the following antibodies: Hsp70 SPA-810, Hsp90 SPA-840, Hsc70 SPA-815, Hsp27 SPA-800 (all from StressGen/Nordic Biosite, Sweden). The blots were incubated with the appropriate secondary antibody (DAKO, Glostrup, Denmark) for 1 h. After further washes, enhanced chemiluminiscence detection was performed according to the instructions of the manufacturer (Pierce, Rockford, IL). After the development of the film, the blots were washed three times in PBS with 0.05% Tween 20 for 5 min. The blots were then incubated with β actin antibody and developed according to the protocol described earlier. Analysis of the spots on the autoradiography was performed using Lotus Graphic software. β actin was used to internally correct the measurements.

2.3. Cell survival

Cell survival after exposure to various stresses was measured with the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. About 16,000 cells were seeded per well in a 96-well plate 24 h before the experiment. Cells were then exposed to one of the following stresses: 10% ethanol for 1 h, 550 μM H_2O_2 for 1 h or a UV-A dose of 1200 J. The wells were washed in Hank's and new medium was added. After 24 h, MTT (Sigma, M2128) was added at 0.5 mg/ml in medium. After 4 h, MTT was removed and isopropanol and HCl were added to dissolve the MTT crystals for 12–16 h. The absorbance at 595 nm was measured. Data were statistically analyzed by Student's *t*-test.

2.4. H_2O_2 decomposition, lipofuscin level and proteasome activities

The measurement of the H_2O_2 decomposition capacity, which is an indirect measure of catalase activity, was performed as described (Spitz et al., 1987; Gill et al., 1998). In brief, 20 μg protein was transferred to an Eppendorf tube, 15 mM H_2O_2 was added and the extent of decomposition was measured over one minute by spectrophotometry at 240 nm. Data were statistically analyzed by Student's *t*-test. Lipofuscin was extracted according to the protocol described (Klikugawa et al., 1995). In brief, cellular lysate was incubated with 10% SDS solution, quenched in a mortar and centrifuged at 105,000*g* for 1 h. Autofluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 435 nm as described (Yin, 1996). Data were statistically analyzed by Student's *t*-test. Three types of proteasome activities, that is, trypsin-like, chymotrypsin-like and caspase-like were determined according to the method described (Bulteau et al., 2000).

3. Results

Basal levels of Hsp90 and Hsc70 in serially passaged human adult skin fibroblasts ASF-1 exposed or unexposed to RMHS are shown in Fig. 1. Considering the level of Hsp in early passage young cells (less than 50% lifespan completed) as 1 A.U., the level of Hsp90 was almost unchanged in cells with 80% lifespan completed followed by a 40% decline in late passage senescent cells. In comparison, RMHS treatment dramatically reduced the level of Hsp90 in both the young and serially passaged senescent cells by 50–85% and more than 98%, respectively (Fig. 1). In the case of constitutive Hsc70, the pattern of age-related change was quite different from that of Hsp90. Although there appeared to be a slight decrease in

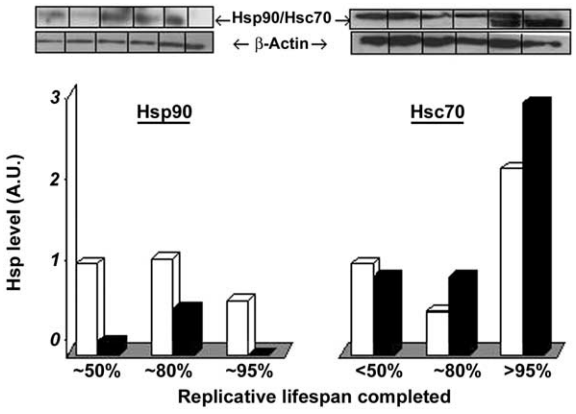

Fig. 1. Western blot-based comparison of basal levels of Hsp90 and Hsc70 in human skin fibroblasts during serial passaging. Open bars: controls. Filled bars: cells exposed to repeated mild heat shocks. Data are presented in arbitrary units (A.U.) taking the intensity of the respective Hsp in early passage (less than 50% lifespan completed) as 1 A.U.

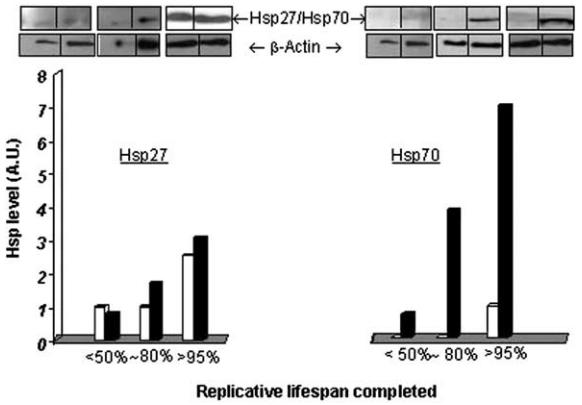

Fig. 2. Western blot-based comparison of basal levels of Hsp27 and Hsp70 in human skin fibroblasts during serial passaging. Open bars: controls. Filled bars: cells exposed to repeated mild heat shocks. Data are presented in arbitrary units (A.U.) taking the intensity of the respective Hsp in early passage (less than 50% lifespan completed) as 1 A.U.

Hsc70 level between early passage and middle-aged cells, this difference was statistically not significant. However, late passage senescent cells showed three equal intensity bands of antibody-detectable Hsc70, which are most likely to be the known isoforms of Hsc70. The cumulative intensity of three Hsc70 bands was more than 2-fold increased in senescent cells as compared with young cells (Fig. 1). A similar trend was observed for RMHS-treated cells where significantly increased levels (3-fold) of Hsc70 were observed in senescent cells. However, of the three Hsc70 bands, the intensity of the high molecular weight band was significantly reduced than the other two bands (Fig. 1).

Serial passage-related and RMHS-related changes in the levels of Hsp27 and Hsp70 are shown in Fig. 2. In the case of Hsp27, whereas untreated control cells showed an increase of about 2.5-fold in late passage cells only; RMHS-treated cells had higher levels of Hsp27 than controls both in middle-aged (2-fold) and senescent cells (more than 3-fold). Differences between unstressed and RMHS cells were most dramatic in the case of the basal levels of Hsp70 (Fig. 2). In this case, since Hsp70 was almost undetectable in unstressed early passage young cells, its level in RMHS cells was considered as 1 A.U. In late-passage control senescent cells, there was an increase in the detectable levels of Hsp70, which were comparable to the early passage RMHS cells. However, RMHS treatment increased the basal levels of Hsp70 by more than 4-fold in middle-aged cells and by more than 7-fold in late passage senescent cells (Fig. 2).

Other effects of RMHS that were observed in this study are summarized in Table 1. These results show that RMHS treatment improved the functional ability of human cells in terms of enhanced stress resistance, increased ability to decompose H_2O_2, increased activities of the proteasome, and reduced accumulation of lipidperoxide product lipofuscin. For example, a 2.5-fold increase (from 5 to 12 mmol/min/mg protein) in the ability to decompose H_2O_2 was observed in the RMHS cells within the first few RMHS treatments. Although the improvement in H_2O_2 decomposition ability decreased with serial passaging, senescent RMHS cells still had about 50% higher ability than senescent control cells (5 versus 7.6 mmol/min/mg protein, respectively). Similarly, a significant increase of 44–95% in early passage cells and of 7–33% in late passage RMHS cells was

Table 1
Effects of RMHS treatment on human diploid skin fibroblasts undergoing aging in vitro

Characteristic	Effect in RMHS-treated cells	
	Early passage cells	Late passage cells
H_2O_2 decomposing capacity	140%	50% increased capacity
Ethanol stress survival	40%	11% increased survival
H_2O_2 stress survival	7%	18% increased survival
UV-A stress survival	17%	5% increased survival
Lipofuscin level	29%	6% decreased level
Caspase-like activity	44%	33% higher activity
Chymotrypsin-like activity	95%	7% higher activity

observed for two of the three types of proteasomal activities, namely chymotrypsin-like and caspase-like (Beedholm et al., unpublished data). Parallel to these observations, the effects of RMHS on reducing the extent of lipofuscin by 29% in young cells and by 6% in senescent cells were also observed. Additionally, RMHS treatment significantly improved the ability of cells to resist other kinds of stress, including ethanol, H_2O_2 and UV-A, as measured by MTT survival assay (Table 1).

4. Discussion

The results presented herein provide further evidence that RMHS treatment has beneficial hormetic effects on a wide variety of biological characteristics of human fibroblasts undergoing aging in vitro. Improved stress resistance and reduced accumulation of lipidperoxide products in aging cells is accompanied by an increase in the proteasomal activity and enhanced ability to decompose H_2O_2. Since HS is the hormetic agent used in these studies, it is important to elucidate any changes in the levels of various Hsp involved in this response. Previous studies reporting age-related changes in the levels of Hsp have normally compared the extent of induction of Hsp after severe heat stress, but none has studied the effects of repeated mild stress. Therefore, the results presented in this study are novel and help to understand the role of Hsp in the maintenance and survival of cells during aging in vitro.

Our studies show that the basal levels of both the constitutive Hsc70 and stress-inducible Hsp70 and Hsp27 proteins increase during serial passaging of human skin fibroblasts even without any HS. A similar increase in the basal level of Hsp22 in aged *Drosophila* (King and Tower, 1999), and Hsp70 in rat kidneys (Maiello et al., 1997) has been reported previously and is taken as the cells' adaptive response to increased intracellular stress during aging. Therefore, it appears that increased levels of Hsp27, Hsc70, and Hsp70 in late passage senescent cells are indicative of their failed attempt to maintain structural and functional ability and to survive for as long as possible. In comparison, exposing these cells to repeated bouts of mild stress stimulates the synthesis of these Hsp, maintains their levels high and helps to improve the functional ability and survival of cells without interfering with their replicative lifespan. However, whereas the difference in the levels of Hsp70 and Hsp90 was statistically significant, the levels of Hsp27 and Hsc70 were statistically similar in normal and RMHS-treated serially passaged cells. Further analysis of the activities and different modes of action of these Hsp and the molecular significance of their increased levels during cellular aging and RMHS treatment is yet to be performed. Also, it will be important to find out the exact nature and significance of the two extra bands of Hsc70 in late passage senescent cells.

In contrast to the increase in the basal level of some Hsp discussed earlier, the basal levels of Hsp90 decreased significantly during serial passaging with and without RMHS treatment. Recently, it was reported that HS treatment reduced the amount of Hsp90 in NIH-3T3 cells (Zhao et al., 2002). Although the exact mechanism for the disappearance of Hsp90 is not fully understood, it has been proposed that Hsp90 during stress binds to partially unfolded proteins and is degraded together with them in a manner similar to what can be observed for Hsp70 after HS (Buchner, 1999). Furthermore, Hsp90 is a powerful modulator of the HS transcription factor Hsf1 activation, and the deletion of Hsp90 has been shown to promote yeast cells' ability to launch a stress response (Harris et al., 2001). Therefore, it is possible that a decrease in the level of Hsp90 during cellular aging and after RMHS treatment is also an adaptive response resulting in the activation of Hsf1, which then stimulates the transcription and translation of other Hsp. Our studies are in progress to find out the kinetics of Hsf1 activation and its modulation by RMHS.

Acknowledgements

This project is a part of the shared cost action programme PROTAGE under the EU Biomed & Health Programme and Quality of Life Projects. Research grants from the Danish Medical Council (SSVF), Danish Research Council (SNF), and Senetek PLC are also acknowledged.

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