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RESEARCH ARTICLE

Differential translocation of heat shock factor-1 after mild and severe stress to human skin fibroblasts undergoing aging *in vitro*

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Abstract Repeated exposure to mild heat shock (HS) has been shown to induce a wide range of health promoting hormetic effects in various biological systems, including human cells undergoing aging *in vitro*. In order to understand how cells distinguish between mild and severe stress, we have investigated the extent of early and immediate HS response by analyzing the nuclear translocation of the transcription factor heat shock factor-1 (HSF1), in serially passaged normal adult human facial skin fibroblasts exposed to mild (41 °C) or severe (43 °C) HS. Cells respond differently when exposed to mild and severe HS at different passage levels in terms of the extent of HSF1 translocation. In early passage young cells there was a 5-fold difference between mild and severe HS in the extent of HSF1 translocation. However, in near senescent late passage cells, the difference between mild and severe stress in terms of the extent of HSF1 translocation was reduced to less than 2-fold. One of the reasons for this age-related attenuation of heat shock response is due to the fact there was a higher basal level of HSF1 in the nuclei of late passage cells, which is indicative of increased intrinsic stress during cellular aging. These observations are consistent with previously reported data that whereas repeated mild stress given at younger ages can slow down aging and increase the lifespan, the same level of stress given at older ages may not provide the same benefits. Therefore, elucidating the early and immediate steps in the induction of stress response can be useful in deciding whether a particular level of stress is potentially hormetically beneficial or not.

Keywords Cellular stress · Heat shock response · Hormesis · Hormetin

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Introduction

The ability of a biological system to respond to stress is an integral component of its survival ability, also known as the property of homeostasis or, more accurately, homeodynamics (Yates 1994). However, the stress response is non-linear, biphasic and hormetic; and its biological consequences can be physiologically variable in being advantageous or harmful depending on the level of the stress (Calabrese and Baldwin 2001; Calabrese et al. 2007). Several lines of investigation have shown that whereas severe and chronic stress is generally harmful, transient and repeated exposures to low level or mild stress strengthen the homeodynamics and enhance survival and longevity of cells and organisms (Rattan 2008; Le Bourg and Rattan 2008; Rattan and Le Bourg 2014). For example, it has been well documented that single or multiple exposures to mild heat shock (HS) have a range of physiologically positive effects in human cells in culture (Rattan 1998). Among these effects are the slowing down of aging *in vitro*, extension of proliferative lifespan, reduced accumulations of damaged proteins, stimulation of the proteasomal activity, improved functionality in terms of migration, differentiation and angiogenesis, and enhanced resistance to other stressors, such as irradiation and ethanol (Verbeke et al. 2001a; Rattan 2004; Nørgaard et al. 2006; Fonager et al. 2002; Rattan et al. 2009). The beneficial effects of mild HS are not limited to cells in culture, and have also been reported for

other organisms, including *Drosophila* (Norry and Loeschcke 2002; Hercus et al. 2003) and *C. elegans* (Olsen et al. 2006). Recently, health beneficial effects of mild HS have also been reported for human beings (Gayda et al. 2012; Kukkonen-Harjula and Kauppinen 2006; Scapagnini et al. 2014). However, it is not yet clear whether there are qualitative and/or quantitative differences in cellular response to mild and severe HS, especially at an early stage of stress detection, which could account for the differences in the biological end-points.

Heat shock response (HSR) is one of the most conserved stress responses throughout evolution; and molecular mechanisms of its transcriptional, translational, and post-translational regulation have been well described (Richter et al. 2010; Verbeke et al. 2001b). An early and immediate step in HSR is the trimerization and translocation of the transcription factor, heat shock factor 1 (HSF1), from the cytoplasm to the nucleus. In unstressed cells, HSF1 is located both in the cytoplasm and in the nucleus. It is maintained as a non-DNA-binding inactive complex both by internal coiled-coil interactions and by stoichiometric binding with HSP90, HSP70 and other chaperones including HSC70, CyP40, Hdj-1, p23, and FKBPs (Richter et al. 2010). The synergistic interaction between these chaperones modulates HSF1 activity by feedback repression. During and after HS, cellular proteins undergo denaturation and/or polyubiquitination and sequester the chaperones capping HSF1. The inactive HSF1 in the cytoplasm becomes free and translocates into the nucleus. HSF has a nuclear localization sequence that is both necessary for its transition from an inactive to an active state and for its nuclear import. HSF1 is activated by trimerisation and subsequent phosphorylation. Several protein kinases, including ErK1-MAP kinase, glycogen synthase kinase-3, protein kinase C, stress activated protein kinase (SAPK)/jun kinase (JNK) and p38 MAP kinase have been shown to phosphorylate HSF1 (Ciocca et al. 2013; Anckar and Sistonen 2011). The active HSF1 binds to the HS responsive element (HSE) present in the promoter region of HS genes, and induces the transcription of several genes, followed by the preferential translation of the so-called heat shock proteins (HSPs), which could be referred to as the late response (Westerheide et al. 2012).

Continuing our investigations on the molecular basis of the hormetic effects of HS, here we describe how normal human skin fibroblasts respond to mild and severe HS in terms of immediate HSR by differential translocation of HSF1, and how the extent of response differs in early passage young cells and late passage near-senescent cells. The results of this initial study will facilitate further investigations on the molecular mechanisms of hormetic and health beneficial effects of mild stress as compared with the negative and harmful effects of severe stress.

Materials and methods

Cell culture

Normal diploid human facial skin fibroblast cell strain, designated FSF-1 (kindly provided by LVMH Research, St. Jean de Braye, France), was initiated from a skin biopsy of an eyelid reduction of a healthy 40-year-old woman. Cells were cultured as described previously (Jørgensen et al. 2014), in an incubator at 37 °C, 95 % relative humidity, and 5 % CO_2, using Dulbecco's Modified Eagle's Medium (DMEM, Biowhittaker, Lonza Inc., USA, BE12-604 F), supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin (Biowhittaker, Lonza Inc., USA, BE17-161E). When near confluent, the cells were trypsinized to be split into new cell culture flasks. FSF-1 cells were serially sub-cultured or passaged at 1:2 or 1:4 split ratio until they stopped dividing, became irreversibly growth arrested and senescent. In order to estimate the proliferative lifespan of FSF-1 cells, 1 or 2 passages (P) were added to the age of the cultures at each subculturing at 1:2 or 1:4 splitting, respectively.

Experimental set-up for stress induction

Before exposure to different experimental conditions, cultures were split into either new flasks, or seeded on microscopy slides with 4 or 8 removable chambers. For HS experiments, FSF-1 cells were exposed to HS by placing them for 1 hr in a pre-heated water bath, with temperatures corresponding to either mild (41 °C) or severe (43 °C) HS, respectively (Kraft et al. 2006; Nielsen et al. 2006). Cells were either harvested for Western blotting or were fixed for immunofluorescence microscopy, immediately after HS treatment (see below).

Cell fractionation and Western-blotting

After exposure to different experimental set-ups, cells were collected by scraping in 1 ml cold PBS on ice. It was followed by a fractionation of the cytoplasm and nucleus using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, USA, Prod# 78833), DNase I, $MgCl_2$ (50 mM) and $CaCl_2$ (13 mM), which were added to nuclear extracts. Protein concentrations were quantified using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). For Western blotting, 20 μg of each sample was loaded (diluted according to protein measurements) onto prefabricated 12 % Bis-Tris SDS-PAGE gels (BIO-RAD Criterion XT, USA, Cat#345-0118), and then electro-transferred to a 0.45 μm nitrocellulose membrane (BIO-RAD, Germany, Cat# 162–0235). Membranes were blocked in PBS containing 4 % non-fat dry milk and then incubated with primary antibody to HSF1 at 1:1000 dilution (Enzo Life Sciences, USA, Cat# ADI-SPA-901). After washing once

with 0.05 % Tween-PBS and 3 times with PBS, membranes were incubated with HRP-conjugated secondary anti-rabbit antibody at 1:500 dilution (DAKO, Denmark, Cat#: P0399), and washed in the same way again. Immunoreactive bands were detected using the chemiluminescence ELC (GE-Healthcare, USA, RPN2232). The quantification was done as a colorimetric density analysis of the band intensity from the Western blot using ImageJ software (http://rsbweb.nih.gov/ij/), and all the corresponding values were normalized to the relative β-actin levels (Sigma-Aldrich, USA, A5441).

Immunofluorescence microscopy

Cells were transferred to 4-well PCA fluorescence slides 48–72 hrs before treatment. After stress exposure, the culture medium was removed and cells were fixed in 2 % paraformaldehyde for 8 min at room temperature, washed 3 times with PBS, permeabilized with 0.1 % Triton X-100 in PBS for 10 min and washed again. The samples were blocked with PBS 4 % milk for at least 30 min at room temperature. Primary antibodies were added at 1:250 dilution for HSF1 (Enzo Life Sciences, USA, ADI-SPA-901), and 1:500 for LC3 (MBL, PM036), and the slides were incubated for 2 hrs at room temperature. After 3 washes with PBS, cells were incubated with secondary antibody (1:500 dilution) Alexa Fluor 488 Goat Anti-Rabbit (Invitrogen, USA, A11008) for 2 hrs at room temperature. After washing again, wells were removed and the slides were mounted with mounting medium containing DAPI for nuclear staining (Vectashield, USA, H-1200). The slides were protected from the light in the fridge at least for 30 min before examining them using a fluorescence microscope.

Results and discussion

In this series of experiments, starting from P-3, serially passaged FSF-1 cells attained a cumulative P level of 65, corresponding to 100 % of proliferative lifespan completed, in a period of about 430 days (Fig. 1). For experiments and data presentation, cells were considered as young at early passages (up to P-14), which is equivalent to 20 % of lifespan completed, and considered as old or near senescent at late passages (P-50 and above) with >80 % of maximum lifespan completed. Figure 1 shows the longevity curve and the phase-contrast microscopic pictures of early passage (P-8) and late passage (P-58) FSF-1 cells, as a typical Hayflick phenomenon of serial passage-associated cellular aging *in vitro* (Hayflick and Moorhead 1961; Rattan 2012). For the first 100 days of serial passaging and until P-40, FSF-1 cells proliferated rapidly at a constant rate of about 1 population doubling per 2.5 days. During this period, cells had the normal morphology as described for young cells, in terms of thin elongated cells,

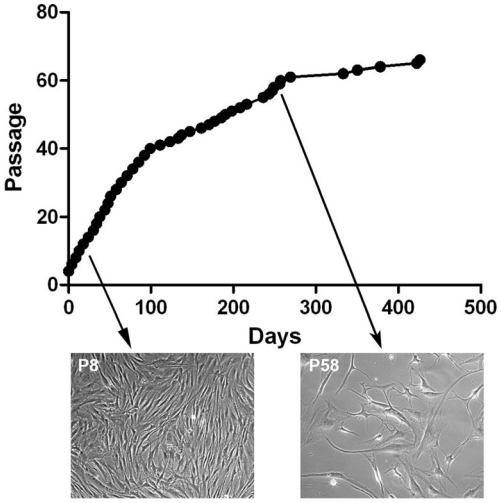

Fig. 1 Longevity curve of serially passaged human facial skin fibroblasts (FSF-1) showing passage level as a function of days in culture until the cells reached a state of replicative-senescence after approximately 350 days. Phase contrast microscopic pictures show early passage cells (P8) and late passage cells (P58), respectively; 10× microscopic magnification

arranged in whorls, and without any appreciable amount of intracellular debris in the form of phase-contrast dense lysosomal residual bodies. However, after about P-40, the growth of FSF-1 cells started to slow down progressively during the subsequent 150 days undergoing 20 more passages, and eventually reaching a permanently growth-arrested state of replicative senescence. Cells in near-senescent state used in this study, had all the well described morphological features, including increased cell size, change of shape from thin, long and spindle-like to flattened and irregular, increased number of vacuoles, highly polymerized actin filaments and disorganized microtubules in the cytoskeleton (Rattan 2012). A frequently used marker for cellular senescence is the senescence-associated β-galactosidase (Dimri et al. 1995), which was also observed to increase from less than 5 % positive cells in early passage cultures to more than 85 % positive cells in late passage cultures (data not shown).

In order to understand the molecular mechanisms of how the biological effects of mild and severe HS vary significantly, we studied the translocation of HSF1 from the cytoplasm to the nucleus, as a well-known early and immediate step in HSR (Verbeke et al. 2001b; Morley and Morimoto 2004; Putics et al. 2008). Figure 2 shows early passage young FSF-1 cells immuno stained for HSF1 after being exposed to either 41 °C or 43 °C for 1 hr, while the controls were kept under standard culturing conditions at 37 °C. Unstressed control cells exhibited very low levels of HSF-1, represented by green fluorescence, both in the cytoplasm and the nucleus. This low level of HSF-1 may be due to the fact that in unstressed cells most of the HSF-1 is sequestered by its interaction with HSP90 and may not be detectable by the antibodies used for immuno cytochemistry. However, in the cells subjected to HS, both

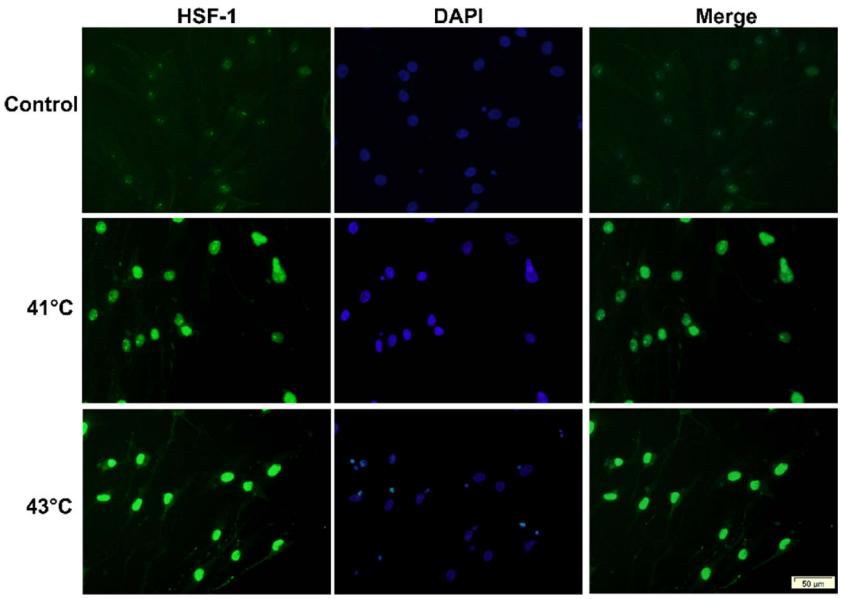

Fig. 2 Nuclear translocation of HSF1 in early passage young FSF-1 cells after exposure to mild (41 °C) or severe (43 °C) heat stress for 1 hr, detected by immunocytochemical staining for HSF1 and DAPI-staining for nuclear DNA, respectively; 40× microscopic magnification; *scale bar*: 50 μm

mild and severe stress, there was a clear cut translocation of the HSF1 from the cytoplasm into the nucleus, which was co-localized with nuclear DNA, as detected by staining with DAPI (Fig. 2).

Although the immunofluorescence pictures clearly show that both mild and severe HS induce the translocation of HSF1 in early passage FSF-1 cells, it is rather difficult to quantify any differences with respect to the extent of translocation by the fluorescence intensity alone. Therefore, for further quantification, Western blot analyses were preformed after separation of the cytoplasm and the nuclei. Figure 3 shows that there was a stress level-dependent increase in the extent of nuclear localisation of HSF1 in early passage cells. In comparison with unstressed control cells at 37 °C, cells exposed to mild HS (41 °C) had approximately 5-fold higher levels of HSF1, while cells exposed to severe HS (43 °C) had about 14-fold higher levels of HSF1 in their nuclei (Fig. 3).

This difference of about 3-fold in the immediate HSR of early passage young human fibroblasts to mild and severe stress is an important novel observation, which can be one of the regulatory steps for the differential biological effects of mild and severe HS reported previously (Kraft et al. 2006; Fonager et al. 2002; Verbeke et al. 2001a). Furthermore, this difference in the extent of HSF1 translocation between mild and severe HS is consistent with the 3-fold differences in the extent of HSP70 synthesis in human skin fibroblasts exposed to either 41 °C or 43 °C HS for 1 hr, reported previously (Fonager et al. 2002). These observations also suggest that FSF-1 cells are able to detect the difference in the intensity of the stress already at the immediate stress response level by means of the transcription factor translocation. As a result, the synthesis of HSPs following this initial response may be regulated and an effective feedback loop involving HSPs may become established affecting the biological end-points. However, direct evidence in support of this interpretation needs to be collected. Moreover, since the activation and translocation of HSF1 by HS is generally dependent on the extent of protein-denaturation caused by HS (Soti and

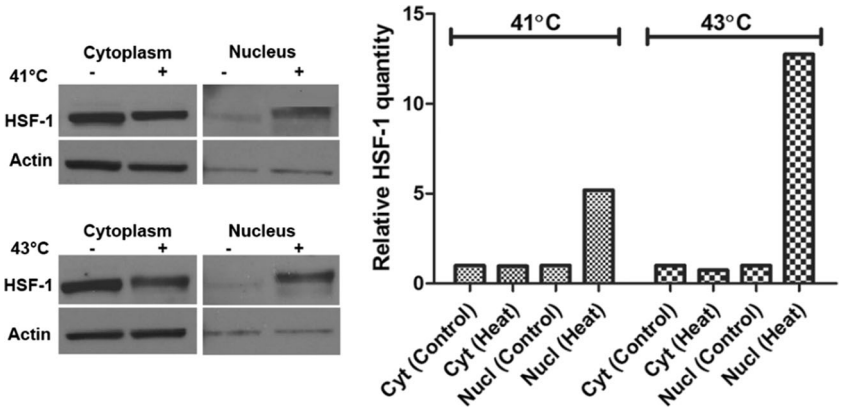

Fig. 3 Western blot analysis of nuclear and cytoplasmic fractions of early passage FSF-1 cells subjected to 41 °C or 43 °C heat stress (indicated by +) or under normal conditions (37 °C). Relative quantification of the HSF1 levels from the WB is represented in the histogram

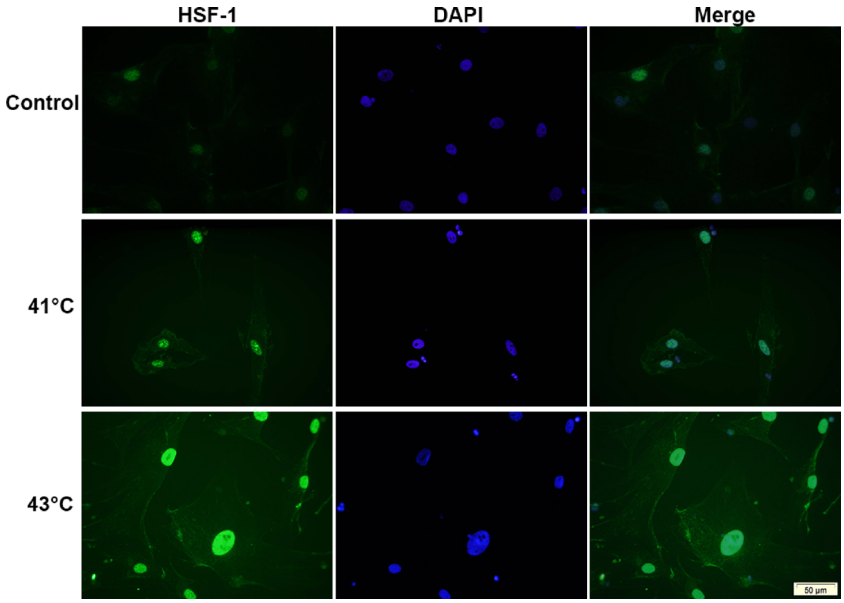

Fig. 4 Nuclear translocation of HSF1 in late passage near-senescent FSF-1 cells after exposure to mild (41 °C) or severe (43 °C) heat stress for 1 hr, detected by immunocytochemical staining for HSF1 and DAPI-staining for nuclear DNA, respectively; 40× microscopic magnification; *scale bar*: 50 µm

Csermely 2007), it will be important to determine the differences in the level of protein denaturation and HSF1 activation caused by mild and severe HS.

Another important variable in HSR is the age of the biological system. For example, it is well known that there is an age-related attenuation of HSR in cells, tissues and organisms (Gutsmann-Conrad et al. 1998; Paglin et al. 2005). Furthermore, it has also been reported that there is an age-related increase in the basal levels of HSF1, which may be an indication of an increase in intrinsic stress levels during aging (Heydari et al. 2000). However, what is not known is whether the ability of old cells to differentiate between mild and severe HS is also compromised or not. Figure 4 shows the microscopy images of immuno stained HSF1 in late passage near senescent FSF-1 cells, which are much more heterogeneous and enlarged as compared with early passage young cells. Even under normal, non-stressful conditions at 37 °C, a small proportion (estimated to be about 10 %) of the cells were HSF1-positive in their nuclei. In addition, highly enlarged size of late passage cells also made it more likely to detect low levels of HSF1 (Fig. 4). Quantification of this difference by Western-blotting showed that the cells exposed to mild HS had 7.5-fold higher levels of HSF1, and the cells exposed to severe HS had 12-fold higher levels of HSF-1, as compared with the controls at 37 °C (Fig. 5). It must, however, be pointed out that the interpretation of WB data from cells in culture must be made with caution since these data do not take into account the heterogeneous and dynamic nature of cell culture populations. This aspect needs further investigations, especially at the level of single cell analysis, and by using more stringent criteria.

Despite above limitations, the results of our studies suggest that with respect to age as a parameter, the difference between mild and severe HS-induced changes in HSF1 was apparently less in late passage near-senescent cells (1.6-fold), as compared with early passage young cells where this difference was more than 3-fold. These results are reflective of the fact that there is an age-related attenuation of HSR (Gutsmann-

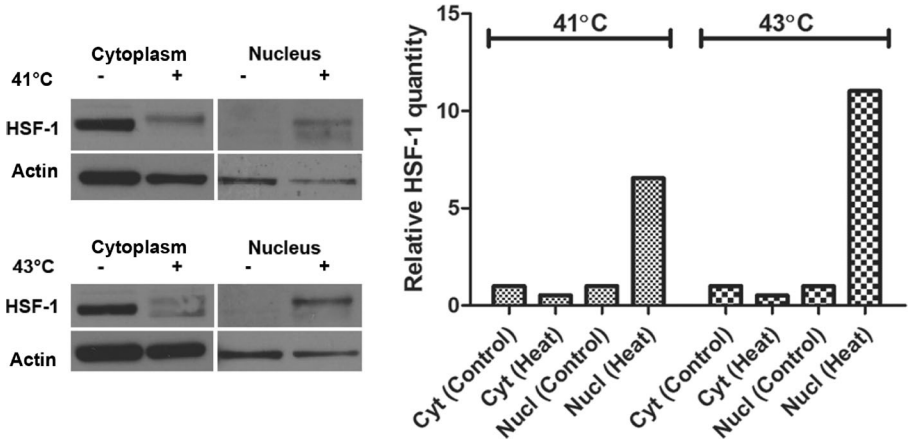

Fig. 5 Western blot analysis of nuclear and cytoplasmic fractions of late passage near-senescent FSF-1 cells subjected to 41 °C or 43 °C (indicated by +) heat stress or under normal conditions (37 °C). Relative quantification of the HSF1 levels from the WB is represented in the histogram

Conrad et al. 1998; Verbeke et al. 2001b; Paglin et al. 2005; Richter et al. 2010), and that the response to mild and severe HS is different at different ages (Hercus et al. 2003; Le Bourg 2005). However, an interesting observation made in the present series of experiments was that the late passage cells did respond to a higher extent when exposed to mild HS as compared to early passage cells, suggesting an over compensatory effect of relatively harmless stress.

These results add new information towards understanding the molecular basis for the non-linear hormetic differences in early HSR to mild and severe stress in human fibroblasts undergoing cellular aging *in vitro*. Whereas our studies on the differential translocation of HSF1 reconfirm the past observations that there is an age-related decline in HSR, these studies also highlight two important and novel points. First, both early passage young cells and late passage near-senescent are able to distinguish between mild HS at 41 °C and severe HS at 43 °C at an early stage by virtue of the extent of HSF1 translocation from the cytoplasm to the nucleus; and second, the ability of late passage near senescent cells to distinguish between mild and severe is reduced, and they are more sensitive to stress. However, it is to be noted that the results presented in this report are based on experiments performed on one cell strain established from a single donor. In order to achieve statistical reliability and to establish the universality of these observations, it will be important to repeat and extend these studies by using different cell types and cell strains established from more donors of different ages.

The implications of these initial results in the context of mild stress-induced hormetic effects are that for obtaining any positive and health beneficial effects of stress, the current biological status of the system must be taken into account. For example, it has been shown that whereas a mild HS given to *Drosophila* at younger ages slows down aging and increases the lifespan, the same level of stress given at older ages can be life shortening (Hercus et al. 2003; Le Bourg 2005). Thus, elucidating the early and immediate steps in the induction of a particular stress response can be useful in deciding whether a particular level of stress is potentially hormetic or not.

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