Mechanisms of Ageing and Development xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

journal homepage: www.elsevier.com/locate/mechagedev

Original Article

A preliminary attempt to establish multiple stress response profiles of human skin fibroblasts exposed to mild or severe stress during ageing *in vitro*

Suresh I.S. Rattan[a,*], Dino Demirovic[a], Carine Nizard[b]

[a] Laboratory of Cellular Ageing, Department of Molecular Biology and Genetics, Aarhus University, Denmark
[b] LVMH Research, St. Jean de Braye, France

ARTICLE INFO

Keywords:
Cellular aging
Hormesis
Fibroblasts
Heat shock
Serum starvation

ABSTRACT

Optimal stress response (SR) is an essential aspect of the property of dynamic homeostasis of all biological systems, including cells in culture. Whereas severe stress can induce the so-called stress-induced premature senescence (SIPS), a model developed by Olivier Toussaint, mild stress can strengthen homeodynamics and can postpone senescence through the phenomenon of hormesis. We have attempted to establish multiple stress response profiles (SRP) of early passage young and late passage senescent human facial skin fibroblasts, FSF-1, exposed to either mild (41 °C) and severe (43 °C) heat shock for 1 h, or to mild (2%) and severe (0%) serum deprivation for up to 48 h. The results obtained show that FSF-1 cells exposed to two different intensities of stress from two different stressors separately have differential SRP to mild and severe stress, which also vary significantly between young and senescent cells. Establishing multiple and differential SRP to mild and severe stress may facilitate distinguishing between the mild stress-induced beneficial hormetic effects and the harmful effects of severe stress.

1. Introduction

A few years ago, we had presented a theoretical strategy for establishing stress response profiles (SRP) as the molecular biomarkers of homeodynamic space and the health status of cells during ageing (Demirovic and Rattan, 2013). The rationale for doing so was based on the fact that optimal stress response (SR) is an essential aspect of the property of dynamic homeostasis of all biological systems (Rattan, 2012a,b, 2013; Rattan, 2014). SR involves the initiation of a series of events for maintenance, repair, adaptation and remodelling (Simmons et al., 2009). There are at least seven main pathways of SR as defined by the nature of the stressor(s) (Demirovic et al., 2014; Demirovic and Rattan, 2013). For example, stressors which cause protein denaturation induce the so-called heat shock response (HSR), whereas oxidative damage-causing stressors induce the anti-oxidative SR mediated by Nrf2, and nutritional deficiency induces the autophagic SR, and so on (Demirovic et al., 2014; Demirovic and Rattan, 2013). Most commonly, each SR is studied and analysed individually and provides detailed information about the kinetics of that specific SR only. However, in order to understand how SR as a crucial component of the biological property of homeostasis and homeodynamics affects health, survival and longevity, it was proposed that all SR pathways should be analyzed simultaneously and a complete SRP should be established under a given condition, such as age-, health- and disease-status, during and after exposure to mild or severe stress (Demirovic and Rattan, 2013).

Here we present the results of our first series of experimental studies on establishing SRP in serially passaged human skin fibroblasts undergoing the Hayflick phenomenon of cellular ageing and replicative senescence *in vitro* (Rattan and Hayflick, 2016). Whereas severe stress can induce the so-called stress-induced premature senescence (SIPS), a model developed by Olivier Toussaint (Debacq-Chainiaux and de Magalhaes 2017; Toussaint et al., 2000), mild stress can strengthen homeodynamics and can postpone senescence through the phenomenon of hormesis (Rattan, 2008a,b; Rattan et al., 2007). We have used two conditions, heat shock (HS) and serum starvation, as representatives of the physical and nutritional stress, respectively. Furthermore, we have analysed SRP under mild and severe levels of stress in order to distinguish between the health beneficial hormetic effects of mild stress as compared with the acute survival responses under severe stress conditions (Kumsta et al., 2017; Pakos-Zebrucka et al., 2016; Rattan, 2008a,b; Rattan and Le Bourg, 2014). Although our study is in many ways preliminary, it is the first of its kind and opens up the possibilities of using SRP as the target for monitoring interventions for maintaining, improving and enhancing health, homeodynamics and longevity.

* Corresponding author at: Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds Vej 10C, DK8000 Aarhus – C, Denmark.
E-mail address: rattan@mbg.au.dk (S.I.S. Rattan).

http://dx.doi.org/10.1016/j.mad.2017.09.005
Received 31 March 2017; Received in revised form 20 September 2017; Accepted 21 September 2017

Table 1
Stress response pathways and their specific protein(s) of interest used to determine each stress response and its intensity[a].

Stress Response	Protein(s) of interest	Antibodies' catalog number	Positive/Negative controls
Heat shock response (HSR)	HSP72	ADI-SPA-800 (Enzo Lifesciences)	Heat shock in water bath at different temperatures
Unfolded protein response (UPR)	GRP78	Ab109659 (Abcam)	Addition of 300 nM Thapsigargin (an intracellular Ca^{2+} pump inhibitor)
Autophagic response	LC3-I/LC3-II	PM036 (MBL Int.)	Presence of lysosomal hydrolase inhibitors (10 μM) Pepstatin A and E64d
DNA-damage response (DDR)	ATRIP	sc-33790 (Santa Cruz BT)	Shortwave UVC (254 nm) exposure for 8 s.
Antioxidant response	HO-1	ADI-OSA-110 (MBL Int.)	Addition of 100 μM H_2O_2 for 10 min.
Sirtuin response	Sirt1	Ab7343 (Abcam)	Addition of 100 μM of Nicotinamide (a noncompetitive Sirt1 inhibitor)
Inflammatory response	NF-κB	NF-κB p105 ab7971 (Abcam)	Induction by addition of Interleukin-6

[a] The specific antibodies' catalog number, and the positive and negative control used to individually induce specific stress response pathway is specified.

2. Materials and methods

2.1. Cell culture and stress exposure

Facial adult skin fibroblast cell strain designated as FSF-1, was established from a healthy 40-yr old woman's eye-lid, at LVMH-Research, St. Jean de Braye, France, and stored frozen in liquid nitrogen at passage 2, as described in detail previously (Demirovic et al., 2015). In brief, FSF-1 cultures were grown and maintained in plastic tissue culture flasks (NUNC, Roskilde, Denmark), in an incubator at 37 °C, 95% relative humidity and 5% CO_2, using Dulbecco's Modified Eagle's Medium, (DMEM; Bio Whittaker, Viviers, Belgium), supplemented with 10% bovine Fetal Calf Serum (FCS; Biological Industries, Beit, Haemek, Israel), and 100 U/mL penicillin/streptomycin (Bio Whittaker, Viviers, Belgium). For sub-culturing, monolayer cultures near confluency (90–95% of the growth surface covered by cells) were trypsinized with 0.25% trypsin-EDTA, and distributed into new cell culture flasks. Cells were serially sub-cultured or passaged at 1:2 or 1:4 split ratio until they stopped dividing, became irreversibly growth-arrested and entered a state of senescence. 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 sub-culturing at 1:2 or 1:4 split ratio, respectively.

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 (Demirovic et al., 2015). 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 (Demirovic et al., 2015).

Serially passaged FSF-1 at different passage levels were exposed to various stress conditions: (i) mild (41 °C) or severe (43 °C) HS by dipping the culture flasks in a pre-heated water bath for 1 h; or (ii) serum starvation (mild starvation at 2%, and severe starvation at 0%) for up to 48 h in the basal DMEM. Normal serially passaged FSF-1 maintained at 37° C in complete DMEM with 10% FCS were used as controls. All experiments were performed in triplicates. HS conditions are considered as mild at 41° C and severe at 43° C based on our earlier series of studies reporting the differential and hormetic effects of mild HS on serially passaged human fibroblasts of different origins (Beedholm et al., 2004; Fonager et al., 2002; Verbeke et al., 2001a,b; Verbeke et al., 2002). Similarly, serum starvation conditions of either 2% serum or 0% serum were considered as mild and severe, respectively, based on our earlier studies (Rattan et al., 2009).

2.2. Cell lysis and western blotting

Cells were harvested after removing the culture medium, followed by washing in PBS, before being scraped in 1 mL ice-cold PBS on ice with a cell scraper. The scraped cells were collected in a 1.5 mL Eppendorf tube and centrifuged at 500 x *g*. The cell pellet was lysed in 200 μL lysis buffer (20 mM Tris-HCl with 150 mM NaCl pH 7.6, 2% Triton X-100, 2 mM phenylmethanesylfonyl in isopropanol, 1 x complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 10 μg/mL DNase). Protein concentrations were determined using Bradford reagent (BioRad, CA, USA) before the equivalent of 20 μg of protein was transferred to a precasted 4–12% polyacrylamide gel (Bio-Rad, CA, USA) containing XT Reducing agent (BioRad, CA, USA) and XT sample buffer (BioRad, CA, USA), and boiled for 5 min at 100 °C. The proteins were separated at 200 V in a high salt containing buffer (either XT-MES or XT-MOPS, both from BioRad, CA, USA) for approximately 45 min. The gels were subsequently equilibrated 10 min in Towbin buffer before they were blotted on to a 0.45 μm nitrocellulose membrane (BioRad, CA, USA) and transferred in Towbin buffer at 200 V for 75 min. The membrane was then blocked in 2% milk in PBS for at least 30 min at room temperature (RT) before it was incubated with antibodies. The details of antibody incubations are specified in the results and discussion part. Primary antibody incubation was done either 1 h at RT or overnight at 4 °C, depending on the most optimal combination. The membrane was washed three times with PBS, before it was incubated with secondary antibody for 1 h at RT. The membrane was again washed three times in PBS before proceeded with chemiluminescence ELC (Amersham, GE Healthcare, USA). An X-ray film (Kodak MedicalFilm) was exposed to the membrane in a dark room, and the film was developed in an AGFA Curix 60 developer.

Table 1 provides the list of antibodies used for detection, and their different specific procedures. The reason for selecting one marker protein among several possible markers representing any specific SR was entirely arbitrary based on the easy availability of the respective antibodies.

2.3. Data analysis, software and equipment

Western blot films were scanned on a Cannon Flatbed scanner, and were analyzed using ImageJ (http://rsb.info.nih.gov/ij/) gel analysis software. All the sample bands were quantified in relation to the control band, and furthermore to the equivalent to their respective actin levels. The data analysis was done in Microsoft Excel 2010-13, and the data presentation was completed in Prism GraphPad.

3. Results and discussion

The results are presented as semi quantification data for the respective protein levels of the various SR pathways. It should be noted that the quantification was made from several WB, and that all protein levels were normalized to the β-actin levels in each sample. However, due to a large heterogeneity in WB pictures, no pictures are shown here, but the representative blots are provided as supplementary material. Therefore, the presented data may be considered as a preliminary attempt to establish multiple SRP of FSF-1 cells exposed to mild or severe stress during serial passaging-related ageing in vitro.