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Review

Slowing down aging from within: mechanistic aspects of anti-aging hormetic effects of mild heat stress on human cells★✪

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Since aging is primarily the result of a failure of maintenance and repair mechanisms, various approaches are being developed in order to stimulate these pathways and modulate the process of aging. One such approach, termed hormesis, involves challenging cells and organisms by mild stress that often results in anti-aging and life prolonging effects. In a series of experimental studies, we have reported that repeated mild heat stress (RMHS) has anti-aging hormetic effects on growth and various cellular and biochemical characteristics of human skin fibroblasts undergoing aging *in vitro*. These beneficial effects of repeated challenge include the maintenance of stress protein profile, reduction in the accumulation of oxidatively and glycoxidatively damaged proteins, stimulation of the proteasomal activities for the degradation of abnormal proteins, improved cellular resistance to other stresses,

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Abbreviations: ERK, extracellular signal-regulated kinase; HS, heat shock; HSF, heat shock factor; HSP, heat shock protein; JNK, c-Jun terminal kinase; PGPH, peptidylglutamyl-peptide hydrolase; RMHS, repeated mild heat stress; sHSP, small heat shock protein; TCP-1, T-complex protein.

and enhanced levels of cellular antioxidant ability. In order to elucidate the molecular mechanisms of hormetic effects of RMHS, we are now undertaking studies on signal transduction pathways, energy production and utilisation kinetics, and the proteomic analysis of patterns of proteins synthesised and their posttranslational modifications in various types of human cells undergoing cellular aging *in vitro*. Human applications of hormesis include early intervention and modulation of the aging process to prevent or delay the onset of age-related conditions, such as sarcopenia, Alzheimer's disease, Parkinson's disease, cataracts and osteoporosis.

The highly complex phenomenon of aging is now reasonably well described at the level of molecules, cells, tissues, organs, organisms and populations. Based on these observations, some general principles of aging can be derived, which can be useful for directing future research and intervention. The three main principles of aging are:

- (1) the life history principle, according to which aging is an emergent phenomenon seen primarily in protected environments which allow survival beyond the natural lifespan, termed essential lifespan, in the wild (Rattan, 2000a; 2000b; Carnes *et al.*, 2003);
- (2) the functional principle, according to which aging is the stochastic and progressive failure of homeostasis/homeodynamics which leads to the impairment in functional ability and an increased proneness to diseases and death (Holliday, 1995); and
- (3) the genetic principle, according to which there are no aging genes evolved specifically to cause aging, age-related failure of maintenance and death. However, the genes which do influence aging and longevity are the so-called longevity assurance genes or virtual gerontogenes (Rattan, 1995), whose normal biological functions may be anything but to "cause" aging.

Unlike some other fields of research, it is integral to aging research that effective means of intervention are found, developed and applied for modulating human aging in order to prevent the onset of age-related diseases and to improve the quality of life in old age. A recent approach in gerontomodulation is based in making use of the fundamental characteristic of living systems, the homeodynamic property of self maintenance and repair, as discussed above.

Since aging is characterized by a decrease in the adaptive abilities due to progressive failure of homeodynamics, it has been hypothesized that if cells and organisms are exposed to brief periods of stress so that their stress response-induced gene expression is stimulated and the related pathways of maintenance and repair are enhanced, one should observe anti-aging and longevity-promoting effects. Such a phenomenon in which stimulatory responses to low doses of otherwise harmful conditions have beneficial biological effects is known as hormesis (Rattan, 2001; Calabrese & Baldwin, 2000b). The paradigm for hormesis is moderate exercise, well known for its beneficial effects, despite the production of potentially harmful biochemical metabolites, such as free radicals, acids and aldehydes.

Stresses that have been reported to delay aging and prolong longevity in various organisms include temperature shock, irradiation, heavy metals, pro-oxidants, acetaldehyde, alcohols, hypergravity, exercise and calorie restriction (Calabrese & Baldwin, 2000a; Le Bourg *et al.*, 2000; Masoro, 2000; Minois, 2000; Cypser & Johnson, 2003; Hercus *et al.*, 2003). Hormesis-like beneficial effects of chronic but mild under-nutrition have been reported for human beings (Raji *et al.*, 1998). It has also been reported that intermittent fasting has beneficial effects on glucose metabolism and neuronal resistance to injury in mice, which may be another example of hormesis (Anson *et al.*, 2003).

During the last few years, research done in our labs has shown hormetic effects of mild

stress on human cells. Using a mild stress regime of exposing serially passaged human fibroblasts to 41°C for 1 h twice a week throughout their replicative lifespan *in vitro*, we have reported a variety of beneficial anti-aging effects (Table 1). It is interesting to note that whereas several age-related alterations, such as accumulation of oxidized proteins (Verbeke *et al.*, 2001a), levels of various heat shock proteins (Fonager *et al.*, 2002), proteasome activities (Beedholm *et al.*, 2004), and stress resistance (Fonager *et al.*, 2002; Verbeke *et al.*, 2002), were affected by repeated mild heat shock (RMHS), there was no increase in the proliferative lifespan of cells (Rattan, 1998; Verbeke *et al.*, 2001a). This has implications in separating the phenomenon of aging from longevity. It appears that the progression of cellular aging *in vitro* as the increased molecular disorder and accumulation of damage can be slowed down without escaping the regulatory mechanisms of cell cycle arrest and replicative senescence. Thus the quality of life of the cell in terms of its structural and functional integrity can be improved without upsetting the mechanisms controlling the replicative lifespan of cells (Rattan, 2004).

Table 1. Effects of repeated mild heat shock on human fibroblasts undergoing aging *in vitro*

Characteristic	Hormetic effect	Reference
Cellular morphology	reduced irregularisation	Rattan (1998)
Cell size	reduced enlargement	Rattan (1998)
Carbonylated protein level	5-40% reduction	Verbeke *et al.* (2001a)
Glycation, furasine level	50-80% reduction	Verbeke *et al.* (2001a)
Glycoxidation level	10-30% reduction	Verbeke *et al.* (2001a)
CML-rich protein level	20-85% reduction	Verbeke *et al.* (2001a)
Lipofuscin pigment level	6-29% reduction	Verbeke *et al.* (2001a)
Reduced glutathione level	3-fold increase	Verbeke *et al.* (2001a)
Oxidised glutathione level	2-fold reduction	Verbeke *et al.* (2001a)
Induction of sugar-induced protein damage	10-fold reduction	Verbeke *et al.* (2002)
H_2O_2 decomposing ability	50-140% increase	Fonager *et al.* (2002)
Survival after H_2O_2 exposure	10-18% increase	Fonager *et al.* (2002)
Survival after ethanol exposure	10-40% increase	Fonager *et al.* (2002)
Survival after UVA exposure	5-17% increase	Fonager *et al.* (2002)
Hsp27 level	20-40% increase	Fonager *et al.* (2002)
Hsc70 level	20% increase	Fonager *et al.* (2002)
Hsp70 level	7-20-fold increase	Fonager *et al.* (2002)
Hsp90 level	50-80% reduction	Fonager *et al.* (2002)
Proteasome activities	40-90% increase	Beedholm *et al.* (2004)
20S proteasome content	no change	Beedholm *et al.* (2004)
19S activator content	no change	Beedholm *et al.* (2004)
11S activator content	increase	Beedholm *et al.* (2004)
11S activator binding	increase	Beedholm *et al.* (2004)

So far, the proof of the principle with respect to the hormetic and beneficial effects of mild stress has been provided, but the exact molecular mechanisms that bring about these effects are yet to be elucidated. Here, we review the available data and discuss our

ongoing studies on understanding the molecular mechanisms responsible for manifesting the hormetic effects of RMHS on human cells.

HEAT SHOCK RESPONSE

Heat shock (HS) response is one of the primordial intracellular defence mechanisms against stressful conditions (Verbeke *et al.*, 2001b). Exposure of cells and organisms to stresses such as high temperature, caloric restriction, exercise, oxidative and osmotic stress, heavy metals, proteasome inhibitors, amino-acids analogues, ethanol, glutathione depletion, calcium ionophores and metabolic poisons induce the cellular stress response leading to the preferential transcription and translation of heat shock proteins (HSP). Extracellular stress from physical, chemical and biological sources and intracellular stress from denatured proteins initiates a series of events starting with signal transduction, activation and nuclear translocation of heat shock factors (HSF), DNA binding of HSF, initiation of HS gene transcription, and preferential translation of HSP which then perform various biological functions discussed below.

Optimal HS response in terms of HSP synthesis and activity is essential for cell survival. In contrast, inefficient and altered HS response has been implicated in abnormal growth and development, aging and apoptosis (Söti & Csermely, 2000; Verbeke *et al.*, 2001b). When a cell encounters a "stressor", modifications of the cytoskeleton, cytoplasmic structures, cell surface morphology, cellular redox status, DNA synthesis, protein metabolism and protein stability occur. Stress generates molecular damage, especially abnormally folded proteins, which can aggregate and initiate a sequence of stress response. The cellular stress response can be viewed as an adaptative or "survival instinct" response for the defence and maintenance of its structural and functional integrity (Verbeke *et al.*, 2001b; Kiang & McClain, 2003). Therefore, any chemical, physical or biological agent that induces this series of events can be considered as a "stressor".

Activation of heat shock factors

The induction of the HS response is through the heat shock transcription factors (HSF) working as molecular links between environmental stresses and the stress response (Kiang & Tsokos, 1998; Verbeke *et al.*, 2001b). The four vertebrate HSF are expressed constitutively and cooperate functionally. HSF1 is a long-lived protein, which is an inactive monomer considered to be a general stress responsive factor. HSF1 is expressed ubiquitously and is activated by mild HS as well as multiple environmental or physiological stresses. HSF2 is a short-lived protein present as an inactive dimer refractory to typical stress stimuli except proteasome inhibitors and is considered to be important during embryogenesis and spermatogenesis. HSF3 is also an inactive dimer and an important co-regulator of HSF1, activated by severe HS and chemical stress. HSF3 may exhibit complex interactions with other transcription factors governing development, growth and apoptosis, such as c-Myc and p53. HSF4 constitutively binds DNA even in non-stressed cells and is preferentially expressed in muscle, brain and pancreas (Verbeke *et al.*, 2001b).

In unstressed cells, HSF1 is both located 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. The synergistic interaction between these chaperones modulates HSF1 activity by feedback repression.

During and after stress, the cellular proteins undergo denaturation and/or polyubiquitination and sequester the chaperones capping HSF1. The inactive HSF1 becomes free

and translocates into the nucleus. HSF1 is activated by trimerization and subsequent phosphorylation (Kiang & Tsokos, 1998). Using electrophoretic mobility shift assay, we have demonstrated that RMHS at 41°C activates HSF1 and facilitates its nuclear translocation and DNA binding in human skin fibroblasts, thus initiating the HS response. No studies have yet been performed on other HSF, and also it is not known whether mild stress activates HSF to the same extent as a severe stress at higher temperatures.

Heat shock proteins

Heat shock proteins (HSP) are present in all cell types and organisms, and the genes encoding them are highly conserved. Based on their molecular masses, and on sequence homology, HSP can be categorised in different families (Verbeke *et al.*, 2001b). HSP have a plethora of functions stretching from being chaperone to acting as proteases. In unstressed cells, HSP are involved in folding, assembly, intracellular localisation, secretion, regulation and degradation of other proteins. Under stressful conditions, when protein folding is disturbed, HSP assist in protein refolding, in protein protection, in cellular protection from protein damage, in dissolving aggregated protein, in sequestering overloaded and damaged proteins into larger aggregates, in targeting damaged proteins for degradation and in interfering with the apoptotic programme. It seems that HSP are able to distinguish between slightly misfolded proteins, which can be refolded, and severely misfolded proteins which should be degraded (Söti & Csermely, 2000). This role of HSP have made them an obvious target of interest in relation to protein-pathies — diseases caused by the misfolding of proteins, such as Alzheimer's and Huntington's diseases.

Typically, HSP function either as oligomers or in a complex with other chaperones, co-chaperones and/or nucleotide exchange factors. In response to HS several different small HSP (sHSP) change phosphorylation status and form large (300–800 kDa) oligomers with ATP-independent chaperone activity (Verbeke *et al.*, 2001b). The sHSP and HSP90 families are known to capture unfolded proteins and create a reservoir of folding intermediates preventing further aggregation. sHSP are also able to induce an increase in cellular glutathione (GSH) levels, which works together with ascorbic acid and coenzyme Q as a kind of redox buffer capacity for the cell, and protects the mitochondrial membrane (Préville *et al.*, 1999). Working together with HSP70 the complex is able to work as a kind of cytoplasmic antioxidant by covering the sensitive sites of the proteins. HSP90 and the sHSP α_2-crystallin are able to stabilize a more active form of the proteasome (Verbeke *et al.*, 2001b).

Members of the HSP90 family constitute 1–2% of cytosolic proteins and have stress-related as well as housekeeping functions. HSP90 stabilize damaged proteins during and after stress. HSP90 interact and either modulate the assembly, the stability and/or the activity of particular cellular proteins such as protein kinases, calcineurin, calmodulin, nitric oxide synthase, telomerase, steroid receptors, oncogenes and transcription factors (Verbeke *et al.*, 2001b). HSP90 is presented as a suppressor of cryptic genetic variations by assisting mutant proteins to maintain a wild type structure and function (Rutherford & Lindquist, 1998). HSP90 and p23 play also a direct role in the regulation of the HS response by modulating the HSF1 activation/deactivation process. HSP90, HSP70, HSP60 and p23 make heterocomplex with a variety of transcription factors and protein kinases involved in mitogenic signal transduction. The major function of this complex may be to fold the client protein and to keep it inactive until it reaches its ultimate location. There is also a potential involvement of HSP70 and HSP90 in DNA replication since members of these families interact with

components of the eukaryotic cell cycle. HSP70, HSP90, HSP27 and TCP-1 are known to bind and stabilize actin, tubulin and the microtubules/microfilament network playing a role in cellular morphology and signal transduction pathways. The HSP60/HSP10 chaperonin system is localized primarily in the matrix space of mitochondria where it assists in folding, refolding and/or elimination of mitochondrial proteins (Kiang & Tsokos, 1998; Verbeke *et al.*, 2001b).

Our studies have shown that the basal levels of both the constitutive HSC70 and stress-inducible HSP70 and HSP27 proteins increase during cellular aging of human skin fibroblasts even without any HS (Fonager *et al.*, 2002). A similar increase in the basal level of HSP22 in aged *Drosophila* (King & 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 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 (Fonager *et al.*, 2002). 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.

In contrast to the increase in the basal level of some HSP discussed above, the basal levels of HSP90 decreased significantly during cellular aging with and without RMHS treatment (Fonager *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.

Some HSP are known to be proteases or to make up the components of a protease system involved in the degradation of the damaged proteins. The unrepairable state of a protein could be signalled to the HSP by the extent of unrepairable modifications, such as carbonylation (Dukan *et al.*, 2000). HSP70 and its cofactors as well as HSC70, HSP90 are involved in the recognition and the degradation of unnecessary and damaged proteins by the proteasome pathway (discussed below). Decreased association of certain proteins with HSP90 and increased association with HSP60/HSP70 lead to their 20S proteasome-mediated degradation. HSP70 has been shown to promote the poly-ubiquitination of damaged proteins. Ubiquitination seems also to be involved in the degradation of unfolded polypeptide by the lysosome. One major mechanism of the lysosomal degradation of proteins is dependent on HSC73 and is responsible for the degradation of a significant amount of the cytosolic protein (Cuervo & Dice, 2000a).

PROTEIN DEGRADATION

One of the main effects of RMHS on human cells is the reduction in the extent of accumulation of oxidatively and glycoxidatively damaged proteins (Verbeke *et al.*, 2000; 2001a). Although this may be due to an increase in cellular resistance of RMHS-treated cells to

glucose and other protein damaging agents (Verbeke *et al.*, 2002), another possibility is the enhanced removal of abnormal proteins by increased degradation. There are three major proteolytic systems in human cells: the proteasomal system, the lysosomal system and the calcium-dependent proteases, calpains, responsible for the turnover of the cytoskeletal and membrane proteins (Cuervo & Dice, 2000b; Grune, 2000; Carrard *et al.*, 2002; Shringaarpure & Davies, 2002).

Proteasome structure, function and relevance to aging and anti-aging

The 20S core proteasome is a multi-catalytic protein complex with three different proteolytic activities and it is responsible for the degradation of most of the oxidized, misfolded and aggregated proteins (Shringaarpure & Davies, 2002). Proteasome is present in the cytoplasm, nucleus and membrane structures, and constitutes approximately 1% of the total cytosolic protein content. A cylindrical 4-ring structure makes up the 20S proteasome with each of the 4 rings containing 7 subunits. The β-rings are the two inner rings, which contain proteolytic β1, β2 and β5 subunits that cleave at acidic, basic, and hydrophobic amino acids, respectively, in a polypeptide chain. The proteolytic activity of the proteasome is restricted to the lumen of the β-rings thereby protecting the cytoplasm against its proteolytic activity. The function of the outer two α rings is to bind the 19S and 11S regulators and control access to the proteolytic lumen of the β-ring. The characteristic of the 20S proteasome to degrade damaged proteins is thought to be due to its ability to recognise hydrophobic structures which become exposed in damaged proteins (Grune, 2000; Carrard *et al.*, 2002).

Age-related decline in the proteasomal activities have been reported for various systems, but the range of the decline varies, and is most dramatic for peptidylglutamyl-peptide hydrolase (PGPH) activity (Wagner & Margolis, 1995; Conconi & Friguet, 1997; Bulteau *et al.*, 2000). Even though many reports affirm a decrease in the proteasome activity during aging, the exact reason for this is not clear at present. The content of 20S proteasome remains unaltered during aging (Bulteau *et al.*, 2000; Brégégére *et al.*, 2003; Beedholm *et al.*, 2004). However, at the mRNA level, gene expression measurements during aging have shown a down-regulation of the α2 and α7 subunits in mice, which was prevented by caloric restriction (Lee *et al.*, 1999). At the protein level, a reduction of the proteasome content in senescent cells may be due to the down-regulation of some of the catalytic subunits of the 20S proteasome and ATPases of the 19S regulatory complex. Over-expression of the catalytic subunits β1 and β5 stimulates the PGPH activity and the activation of the expression of several other subunits, suggesting a common regulation of the expression (Chondrogianni *et al.*, 2003). A more general study, using two dimensional (2D)-gel analysis of proteins in cultured fibroblasts from healthy donors of different ages, has reported a decrease of the proteasome subunits α-3 and α-5 (Boraldi *et al.*, 2003). Another reason suggested for the age-related decline in proteasomal activities is the post-translational modifications, such as oxidation, glycation and glycoxidation (Bulteau *et al.*, 2000; Carrard *et al.*, 2002; Carrard *et al.*, 2003). In our laboratory, using the phage display method of antibody isolation, we have produced antibodies against the glycated α7 subunit of the proteasome, whose levels appear to increase significantly during aging of human cells (Gonzalez-Dosal *et al.*, in preparation).

Our studies on the hormetic effects of RMHS have shown that human skin fibroblasts exposed to RMHS had 40 to 95% increase in proteasomal activities in early and mid-passage cells, without any accompanied increase in the 20S proteasomal content (Beedholm *et al.*, 2004). Furthermore, we have observed that this increase in pro-

teasomal activities was related to a significant increase in the amount of the proteasome activator 11S. The increase of the 20S may be due to an increase in its transcription and translation of 11S activator, an increase in its binding to the 20S proteasome, and a higher level of HSPs in RMHS-treated cells. Although we have not yet determined the extent of transcription, it has been observed that the amount of 11S activator bound to the 20S proteasome was significantly higher in RMHS-treated cells (Beedholm *et al.*, 2004). Such an increased binding makes it possible for the RMHS-treated cells to activate the proteasome faster than the unstressed cells. However, difference between RMHS-treated and control cells disappear in late passage senescent cells. It, therefore, seems that the positive effect of RMHS on proteasome activities are happening when the cells are young and middle-aged, and the beneficial biological consequences of these effects (for example, reduced cell enlargement and reduced levels of abnormal proteins) are carried over to senescent cells.

Lysosomal protein turnover

Lysosome is the other major cellular proteolytic system affected by aging. The HSC73 specific lysosomal-proteolytic-pathway is inhibited in senescent fibroblasts (Cuervo & Dice, 2000b; Hallén, 2002; Dröge, 2004). Accumulation of lipofuscin, which is an aggregate of oxidized proteins and lipids, affects the lysosomal activities (Terman & Brunk, 1998; Terman *et al.*, 1999). Other typical cellular inclusions in senescent cells contain over- aggregated proteins as well as chaperones and proteasome components as if both chaperones and proteases have capitulated in face of various insults. A decline in HSF and HSP activity, if not always a decline in their expression, and decrease in the activities of antioxidant enzymes are thought to underlie human neurodegenerative diseases. This is because imbalances of the cellular redox status and lack of chaperone activity promote protein aggregation and favour the development of aging-linked pathologies including cataract, polyglutamine-related-disorders or other neurodegenerative diseases as well as cancer (Söti & Csermely, 2000; Verbeke *et al.*, 2001b; Söti *et al.*, 2003). Severe stress may also promote some of these pathologies more directly by a transcription pathway. Accumulation of oxidized and aggregated proteins could be responsible for the increase in the constitutive expression of some HSPs such as HSP22, HSC70 and HSP70 observed in aged animals, especially in tissues formed by post-mitotic cells exposed to stress for a long period of time (King & Tower, 1999). We plan to undertake studies on the effects of RMHS on lysosome-mediated protein degradation during cellular aging.

SIGNAL TRANSDUCTION

Little is known about the exact molecular details of the signaling pathways involved in HS response during aging. However, recent studies indicate the crucial role of the stress activated protein kinases c-Jun terminal kinase (JNK) and p38, and mitogen activated protein kinase (MAPK), especially the extracellular signal-regulated kinase (ERK). They can be activated by many different stimuli, including growth factors, cellular stress, cytokines, hormones and cell–cell contact. Their activation involves dual phosphorylation on serine and threonine residues in the activation loop. These activated kinases are then able to phosphorylate other substrates, most of which are transcription factors, but also other protein kinases, phospholipases and cytoskeleton-associated proteins (Widmann *et al.*, 1999).

All three kinases are activated within minutes by HS, but by different pathways. ERK is activated through auto-phosphorylation (activation) of the epidermal growth factor (EGF)

receptor, and it has been shown that the phosphorylation of ERK is not dependent upon EGF (Lin *et al.*, 1997). JNK activation proceeds through the inactivation of a phosphatase which normally inactivates JNK (Meriin *et al.*, 1999). In the case of p38 the upstream MAPK-kinase-kinase (Ask1) is released from an inhibitor GSTM-1 (glutathione *S*-transferase Mu1-1) upon HS (Dorion & Landry, 2002).

There are many interactions between MAPK and HSP. For example, JNK has been shown to phosphorylate and stabilize HSF1 (Park & Liu, 2001). But overexpression of HSP72 is shown to inhibit HS-induced JNK-activation (Meriin *et al.*, 1999). Similarly, p38 phosphorylates MAPKAPK2 which phosphorylates HSP27, which then can affect actin dynamics in the cell (Dorion & Landry, 2002).

With respect to aging, it is known that ERK1 and ERK2 are expressed at the same levels in young and senescent cells, but only ERK1 is phosphorylated by serum stimulation in senescent cells (Lorenzini *et al.*, 2002; Suh, 2002). The activation of JNK is reported to either decline (Adler *et al.*, 1996) or stay unaltered (Volloch *et al.*, 1998) in human fibroblasts undergoing aging *in vitro*. Recently, it has been reported that increasing the JNK activation in *Drosophila* alters their gene expression, enhances their resistance to oxidative stress and increases their longevity (Wang *et al.*, 2003). Our studies are in progress with respect to the analysis of the signal transduction pathways and determining alterations in the phosphorylation and dephosphorylation states of ERK, JNK and p38 MAP-kinases as a measure of cellular responsiveness to mild and severe heat stress. Preliminary results show that already after 5 min at 41°C, there is a 1.5 to 2-fold increase in ERK, p38 and JNK activation in young cells, which increases further to 3-fold activation after 60 min. Further studies will elucidate whether RMHS causes or prevents any age-related shift in the balance of these signaling pathways, which could explain age-related changes in cellular responsiveness and proneness to apoptosis.

FUTURE PERSPECTIVES

Beneficial hormetic effects of RMHS on human cells appear to be facilitated by reducing protein damage and protein aggregation by activating internal antioxidant, repair and degradation processes. HSP are involved in preventing the accumulation of highly damaged proteins during aging since they govern both the repair of weakly damaged proteins and the catabolism of highly damaged proteins. Thus, hormetic pathways are suggested to activate several key proteins involved in the stress response. Therefore, studying age-related and hormesis-related changes in cellular proteomics and in post-translational modifications, such as phosphorylation, oxidation and nitrosylation, leading to altered activity and stability of proteins is the next essential step.

In this context, 2D-gel electrophoresis is a powerful and promising method which can resolve post-translationally modified proteins from their non-modified form. In the last few years instruments and software have been developed for detection and discrimination of multicolour fluorescent signals, and it is therefore possible to do quantitative comparisons of 2D-gel spot analysis. Furthermore, the multicolour fluorescent imaging system in combination with immunochemical blotting methods provides a possibility to simultaneously compare the amount and post-translational modifications of proteins of interest, for example HSP, proteasomal subunits, cytoskeletal components, and cell-type specific proteins. Additionally, it is also important to find out the cellular energy costs of stress-induced hormesis, in terms of ATP synthesis and utilisation, which may be the rate

limiting factor that distinguishes between mild and severe stress. Our studies are in progress in this respect.

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