REJUVENATION RESEARCH
Volume 7, Number 1, 2004

Hormetic Mechanisms of Anti-Aging and Rejuvenating Effects of Repeated Mild Heat Stress on Human Fibroblasts *in Vitro*

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ABSTRACT

The phenomenon of hormesis is represented by mild stress-induced stimulation of maintenance and repair pathways, resulting in beneficial effects for cells and organisms. 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 effects of RMHS include the maintenance of the stress protein profile, reduction in the accumulation of oxidatively and glycoxidatively damaged proteins, stimulation of the activities of the proteasome and its 11S activator, improvement in cellular resistance to ethanol, hydrogen peroxide, and ultraviolet rays, and increased antioxidative activity of the cells. We have also reported that RMHS prolongs the lifespan of *Drosophila*. Others have reported anti-aging and life prolonging effects of a wide variety of so-called stressors, such as pro-oxidants, aldehydes, calorie restriction, irradiation, heat shock, and hypergravity. Although molecular mechanisms of hormesis are yet to be elucidated, there are indications that relatively small hormetic effects become biologically amplified, resulting in significant improvement of cellular and organic functions and survival. Hormesis, therefore, can be an effective approach for modulating aging, for preventing or delaying the onset of age-related diseases, and for improving the quality of life in old age.

INTRODUCTION

SINCE AGING IS CHARACTERIZED by a decrease in the adaptive abilities and a progressive failure of maintenance and repair mechanisms, it has been suggested that if cells and organisms are exposed to brief periods of stress so that their stress response induced gene expression is upregulated and the related pathways of maintenance and repair are stimulated, we should observe anti-aging, health improving, and longevity promoting effects. The phenomenon in which stimulatory responses to low doses of otherwise harmful conditions improve the functional ability of cells and organisms is known as hormesis. However, the harmful effects of high doses have long shadowed the hormetic effects of low-level stress.[1,2] Although most of the work on hormesis has been driven by pharmacological and toxicological studies,[2,3] applying hormesis in aging research and therapy is a relatively recent development.[4–9] The paradigms for the applicability of hormesis in modulating aging and longevity are the well documented beneficial effects of moderate exercise[10,11] and calorie restriction.[12–14]

During the past few years, research done in our laboratory has shown hormetic effects of

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mild heat shock on human skin fibroblasts and in *Drosophila melanogaster*. Using a mild stress regime of exposing serially passaged human fibroblasts to 41°C for 1 hour twice a week throughout their replicative lifespan *in vitro*, we have reported several beneficial anti-aging effects. These effects include the maintenance of youthful morphology, reduced accumulation of damaged proteins,[7,15–17] increased levels of various heat shock proteins, increased antioxidative abilities, and increased resistance to ethanol, hydrogen peroxide, and UV-A irradiation,[18] and increased activities of the proteasome and its 11S activator.[19] In the case of *Drosophila*, exposure of young flies to four rounds of mild heat shock at 34°C significantly increased the average and maximum lifespan of female flies and increased their resistance to potentially lethal heat stress.[20] Similarly, anti-aging hormetic effects of heat shock and other stresses such as hypergravity, irradiation, population density, physical injury, ethanol, hyperoxia and pro-oxidants on various organisms have been reported by several groups.[9] Although the exact molecular mechanisms for the hormetic effects of various stresses are yet to be elucidated, the mechanisms responsible for bringing about the range of hormetic effects of repeated mild heat stress (RMHS) are increasingly becoming clear. What follows is a brief discussion of mild heat shock induced pathways of stress protein synthesis, signal transduction, and proteasomal activation, which are potentially applicable for developing effective means of aging intervention and prevention.

HEAT SHOCK RESPONSE

Heat shock (HS) response is one of the primordial intracellular defense mechanisms against stressful conditions.[21] 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 induces the cellular stress response leading to the preferential transcription and translation of heat shock proteins (HSP). Extracellular stress and intracellular stress from denatured proteins initiate 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.[22] In contrast, inefficient and altered HS response has been implicated in abnormal growth and development, and in aging and apoptosis.[21,23] 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 stress response sequence. The cellular stress response can be viewed as an adaptive or survival response for the defense and maintenance of its structural and functional integrity.[21,24] Therefore, any chemical, physical, or biological agent that induces this series of events can be considered as a stressor.

Signal transduction

Signaling pathways involved in HS response are still largely unknown. However, some kinases in the stress pathways, such as stress-activated protein kinase (SAPK), c-Jun terminal kinase (JNK or SAPK1), and p38 (SAPK2), are suggested to play an important role. HS activates within minutes the major signaling pathways involving mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and SAPK.[25,26] These kinases are involved in both survival and death pathways in response to other stresses and may, therefore, contribute significantly to the HS response.[27] Activation of p38 occurs very early during stress and leads to the phosphorylation of HSP27. It is triggered by a highly specific HS sensing pathway and requires the activation of upstream kinases such as the MAPKK, MKK3/6, and the MAPKKK apoptosis signal-regulating kinase-1 (ASK1).[28] HS is also thought to activate and thus phosphorylate the epidermal growth factor (EGF) receptor in an agonist-independent

way.[26] HS has also been shown to phosphorylate constitutive nitric oxide synthase at tyrosine residues and increase fas/CD95 expression on the cell membrane,[29] in addition to induce increased $[Ca^{2+}]_i$, $[Na^+]_i$, pHi, cAMP cellular levels, and inositol 1,4,5-trisphosphate.[22]

It has been suggested that JNK is preferentially associated with the protective effects of HS against severe stress.[30] A major mechanism for HS-induced JNK appears to be the direct inhibition of the JNK phosphatase that normally inactivates JNK. In the absence of this phosphatase, the basal activity of MAPKK4 (MKK4) is sufficient to activate JNK.[28] An early and transient activation of the JNK and p38 pathways is usually associated with survival and differentiation, whereas a late and sustained activation might point to apoptosis.[26] Therefore a balance between the (apoptotic) JNK and p38 pathways and (survival) ERK pathways, and their interplay, determine whether a cell exposed to HS will die, or survive and become stress tolerant.[27] We are now undertaking detailed analysis of the signal transduction pathways in order to determine alterations in the phosphorylation and dephosphorylation states of ERK, JNK, and p38 MAP-kinases as a measure of cellular responsiveness to mild and severe HS. Preliminary results obtained in our ongoing studies show that after 5 minutes HS at 41°C, there is a 1.5 to 2-fold increase in ERK, p38, and JNK activation in young cells, which increases to a 3-fold increase after 60 minutes. Further studies are in progress to find age-related changes in RMHS-related activation of the above signaling pathways in human cells.

Activation of heat shock factors

The HS response is induced through the heat shock transcription factors (HSF) working as molecular links between environmental stresses and the stress response.[21,22] The four vertebrate HSF are expressed constitutively and cooperate functionally. HSF1 is a long-lived protein, an inactive monomer considered to be a general stress responsive factor which 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 unstressed cells and is preferentially expressed in muscle, brain, and pancreas.[21]

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. HSF have a nuclear localization sequence that is both necessary for the transition of HSF from the inactive to active state and for nuclear import. HSF1 is activated by trimerization and subsequent phosphorylation.[22] Using an 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 (Fonager et al., unpublished data). In the nematode *Caenorhabditis elegans*, reducing HSF1 activity has been reported to accelerate tissue aging and shortening of lifespan, whereas overexpression of HSF1 resulted in the extension of lifespan.[31] Although levels of trimers, extent of oligomerization, nuclear translocation, and phosphorylation of HSF1 are unaltered in hepatocytes isolated from old rats,[32] 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 (HSP)

Genes encoding HSP are highly conserved. Many of their products can be assigned to families on the basis of sequence homology and molecular weight. In mammals, many HSP

families comprise multiple members that differ in inducibility, intracellular localization, and function.[21] HSP are known to play diverse roles as chaperones and/or 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 have been shown to assist in protein refolding, to protect cellular systems against protein damage, to dissolve protein aggregates to some extent, to sequester overloaded and damaged proteins into larger aggregates, to target damaged proteins to degradation, and to interfere with the apoptotic program. Chaperones and proteases can recognize the same protein substrates and the abundance of both types of proteins suggests that HSP are able to distinguish between those proteins that can be refolded and those fated to enter the proteolytic pathway.[24,33]

Some HSP are known to be chaperones and are involved in the renaturation of unfolded proteins. Chaperones recognize and bind to other proteins when they are in non-native conformations and are exposing hydrophobic sequences. Their role is to minimize the aggregation of non-native proteins formed during stress. Typically, chaperones function as oligomers, if not as a complex of several different chaperones, co-chaperones, and/or nucleotide exchange factors.[34] In response to heat and oxidative stresses, different small HSP (sHSP) either become phosphorylated or dephosphorylated. Depending on their phosphorylation status, sHSP form large (300–800 kDa) and active oligomers having an ATP-independent chaperone activity. The sHSP and HSP90 families capture unfolded proteins and create a reservoir of folding intermediates preventing further aggregation. Subsequently, HSP70 and HSP60 families, helped by co-chaperones, bind to the stabilized unfolded proteins in the cytosol, mitochondria, and endoplasmic reticulum and attempt to restore the structure of proteins in a cycle driven by ATP hydrolysis. If the target protein is damaged by post-translational modifications, it could be repaired by specific cellular systems before refolding, but such systems exist for only a few types of damage.[21] In neurodegenerative diseases related to polyglutamine expansion, cotransfection of several chaperones and co-chaperones have significantly reduced the formation of aggregates.[21]

Acting as a molecular chaperone, HSP protect many different systems involved in maintenance of cellular functions. Small HSP induce an increase of the cellular GSH level leading to the protection of the mitochondrial membrane potential during stress.[35] HSP70 contains a novel nuclear localization signal in its C-terminal domain, implying a role for HSP70 in the regulation of nuclear proteins and transcription factors such as HSF. Members of HSP70 and HSP90 families are associated with the centrosome, suggesting an involvement in microtubule nucleation or in centrosome assembly. The protection of protein synthesis during stress, called translational thermotolerance, is due to the association of HSP72 with ribosomal subunits in polysomes of thermotolerant cells. Some chaperones such as the sHSP α_2-crystallin and HSP90 could stabilize a more active conformation of the proteasome.[21]

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 interacts with and modulates 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.[21] HSP90 is presented as a suppressor of cryptic genetic variations by assisting mutant proteins to maintain a wild type structure and function.[36] HSP90 and p23 also play a direct role in the regulation of the HS response by modulating the HSF1 activation/deactivation process. Since HSP90 exists in homeostasis with intracellular hormone receptor and HSF1, it could be hypothesized that steroid hormones activate the HSF by altering this homeostasis. 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 with 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.[21,22]

Our studies show 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.[18] A similar increase in the basal level of HSP22 in aged *Drosophila*[37] and HSP70 in rat kidneys[38] 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 is 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.[18] 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 decreases significantly during cellular aging with and without RMHS treatment.[18] 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.[39] Furthermore, HSP90 is a powerful modulator of the activation of HSF1, and the deletion of HSP90 has been shown to promote yeast cells' ability to launch a stress response.[40] 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 damaged proteins. The unrepairable state of a protein could be signaled to the HSP by the extent of unrepairable modifications, such as carbonylation.[41] HSP70 and its cofactors, as well as HSC70 and HSP90 are involved in the recognition and the degradation of unnecessary and damaged proteins by the proteasome pathway. Decreased association of certain proteins with HSP90 and increased association with HSP60/HSP70 leads to their 20S proteasome-mediated degradation. HSP70 has been shown to promote the polyubiquitination 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.[42]

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.[15,16] Although this may be due to an increase in cellular resistance of RMHS-treated cells to glucose and other protein damaging agents,[17] another possibility is the enhanced removal of abnormal proteins by increased turnover. The bulk of proteolysis is carried out by the ubiquitin-proteasome system in eukaryotes. The proteasome is a multisubunit, multicatalytic proteinase complex, also known as multicatalytic proteinase (MCP). Oxidized proteins are preferentially degraded by the 20S proteasome in an ATP-independent manner, whereas the proteins marked by covalently attached ubiquitin are degraded in an ATP-dependent way by the 26S proteasome, which is ubiquitous among eukaryota, archaebacteria, eubacteria, and prokaryota.[43–45] The eukaryotic proteasome is present both in the nucleus and in the cytoplasm and constitutes approximately 1% of the total content of cytosolic protein. Polypeptides to be degraded are covalently at-

tached to ubiquitin, which is itself an extremely conserved and heat-inducible HSP. The substrates for the proteasome can be categorized as either misfolded, denatured, or otherwise damaged proteins, or perfectly healthy proteins which have to be removed for normal functioning of the cell, such as cell cycle control, protein quality control, apoptosis, and antigen presentation. During aging, there is a decline in the activities of the proteasome, including decreased activity of the proteasome towards artificial peptide substrates as well as the ability to preferentially degrade oxidized proteins.[46–49]

We have found that human skin fibroblast cells exposed to RMHS had 20–100% increased proteasome activity, without any accompanying increase in the 20S proteasomal content.[19] Furthermore, we have observed that this increase in proteasomal activities was related to a significant increase in the amount of the proteasome activator 11S, which is an adaptor between the 20S proteasome and some of the chaperones in the cytosol. The increase of 20S may be due to an increase in its transcription and translation of 11S activator, an increase in its binding to the 20S proteasome, or a higher level of HSP 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.[19] Such increased binding makes it possible for the RMHS-treated cells to activate the proteasome faster than the unstressed cells.

Lysosome is the other major cellular proteolytic system affected by aging. The HSC73-specific lysosomal proteolytic pathway is inhibited in senescent fibroblasts.[42,50] Accumulation of lipofuscin, which is an aggregate of oxidized proteins and lipids, affects the lysosomal activities.[51,52] 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 a decrease in the activities of antioxidant enzymes are thought to underlie human neurodegenerative diseases. Imbalances of the cellular redox status and lack of chaperone activity promote protein aggregation and favor the development of aging-linked pathologies including cataract, polyglutamine related disorders, or other neurodegenerative diseases, as well as cancer.[21,33] 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 HSP 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.[37] However, no studies have yet been done on the effects of RMHS on lysosome-mediated protein degradation.

CONCLUSIONS

Anti-aging hormetic effects of mild HS 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. Cellular resistance to stress has been correlated with natural longevity, supporting the view that the machinery of the cellular stress response is functionally important in aging.[53–55]

Indeed, hormesis leads to the maintenance of the HS response during aging and the concomitant transitory and moderate overexpression of various HSP in *C. elegans* is greatly beneficial.[56,57] Exposure of various lines of *D. melanogaster* to mild heat stress has been shown to increase their lifespan, albeit to varying extents under different conditions.[20,58–61] However, this hormetic effect is not simply due to increased levels of HSP, as further studies have shown that genetic manipulations of *Drosophila* leading to increased HSP70 levels do not necessarily lead to longevity extension.[59,60] Similarly, although heat resistance is almost always associated with lifespan extension in *C. elegans* and *Drosophila*,[62–65] selection of cold-resistant

Drosophila lines did not lead to their extended longevity at normal temperature.[66]

Thus it is clear that the relationship between stress resistance/stress exposure and aging/lifespan is highly complex and is dependent on multiple factors, such as the nature of the stress, the genetic background of organisms, gender, reproductive history, and environmental conditions. Similarly, constitutive overexpression of individual HSP may be toxic for organisms because a chaperone works with a complex of activatory and inhibitory co-chaperones and if some are present in excess, or without others, chaperone sequestration, protein aggregation, retardation in cell growth, and enhanced apoptosis can occur.[21,67]

In any case, age-dependent regulation of HS response may implicate a common protective mechanism against deleterious effects of aging, which may be amenable to modulation through hormesis. Although hormetic effects are normally observed to be quite moderate (20–50% stimulation), when studied at the level of an individual biochemical step, often the final biological outcome, such as stress-tolerance and survival, is much larger and significant than expected.[2,18] This suggests that hormesis is also involved in the *biological amplification* of adaptive responses leading to improvement in overall cellular functions and performance. Exercise is a good example of the biological amplification of beneficial effects of mild stress, where not only do the specific muscle targets gain benefit, but improvements in the immune system, cardiovascular system, sex hormones, libido, and mood are also well documented.[10,68] That is why the use of hormesis in slowing down or preventing age-related loss of neurons, muscles, and bones is considered a real possibility.[2,8]

There are, however, several issues that remain to be resolved before hormesis can be widely used for modulating aging and preventing the onset of age-related impairments and pathologies. These include: how to establish biochemical and molecular criteria for determining the hormetic levels for different stresses; how to identify differences and similarities in stress response pathways initiated by different stressors; how to quantify the extent of various stress responses; how to determine the interactive and pleiotropic effects of various stress response pathways; how to adjust the levels of mild stress for age-related changes in the sensitivity to stress; and how to determine the biologic and evolutionary costs of repeated exposure to stress. Resolution of these issues requires much more research on hormesis than at present. But the proof of the principle that hormesis is a promising anti-aging and rejuvenating approach has already been provided.

ACKNOWLEDGMENTS

The author wishes to thank the Danish Medical Council (SSVF), Danish Research Council (SNF), and Senetek PLC for research grants.

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Received: January 23, 2004
Accepted: February 20, 2004

This article has been cited by:

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