Age and attenuation of exercise-induced myocardial HSP72 accumulation

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Abstract

Overexpression of heat shock protein (HSP)72 is associated with cardioprotection. Hyperthermia-induced HSP72 overexpression is attenuated with senes-cence. While exercise also increases myocardial HSP72 in young animals, it is unknown whether this effect is attenu-ated with aging. Therefore, we investigated the effect of aging on exercise-induced myocardial heat shock factor (HSF)-1 activation and HSP72 expression. Male Fischer-344 rats (6 or 24 mo) were randomized to control, exercise, and hyperthermic groups. Exercise consisted of 2 days of tread-mill running (60 min/day, ~75% maximal oxygen consump-tion). Hyperthermia, 15 min at ~41°C (colonic temperature), was achieved using a temperature-controlled heating blan-ket. Analyses included Western blotting for myocardial HSP72 and HSF-1, electromobility shift assays for HSF-1 activation, and Northern blotting for HSP72 mRNA. Exercise and hyperthermia increased (P ~0.05) myocardial HSP72 in both young (~3.5- and 2.5-fold, respectively) and aged (~3-and 1.5-fold, respectively) animals. Both exercise and hyper-thermic induction of HSP72 was attenuated with age. Myo-cardial HSF-1 protein, HSF-1 activation, and HSP72 mRNA did not differ with age. These data demonstrate that aging is associated with diminished exercise-induced myocardial HSP72 expression. Mechanisms other than HSF-1 activation and transcription of HSP72 mRNA are responsible for this age-related impairment

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Age and attenuation of exercise-induced myocardial HSP72 accumulation

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Overexpression of heat shock protein (HSP)72 is associated with cardioprotection. Hyperthermia- induced HSP72 overexpression is attenuated with senes- cence. While exercise also increases myocardial HSP72 in young animals, it is unknown whether this effect is attenu- ated with aging. Therefore, we investigated the effect of aging on exercise-induced myocardial heat shock factor (HSF)-1 activation and HSP72 expression. Male Fischer-344 rats (6 or 24 mo) were randomized to control, exercise, and hyperthermic groups. Exercise consisted of 2 days of treadmill running (60 min/day, ~75% maximal oxygen consumption). Hyperthermia, 15 min at ~41°C (colonic temperature), was achieved using a temperature-controlled heating blanket. Analyses included Western blotting for myocardial HSP72 and HSF-1, electromobility shift assays for HSF-1 activation, and Northern blotting for HSP72 mRNA. Exercise and hyperthermia increased ($P < 0.05$) myocardial HSP72 in both young (>3.5- and 2.5-fold, respectively) and aged (>3- and 1.5-fold, respectively) animals. Both exercise and hyperthermic induction of HSP72 was attenuated with age. Myocardial HSF-1 protein, HSF-1 activation, and HSP72 mRNA did not differ with age. These data demonstrate that aging is associated with diminished exercise-induced myocardial HSP72 expression. Mechanisms other than HSF-1 activation and transcription of HSP72 mRNA are responsible for this age-related impairment.

stress proteins; cardioprotection; heart; heat shock protein

AGING IS A MULTIFACTORIAL PROCESS resulting in damage to molecules, cells, and tissues. Eventually, this damage exceeds the capacity of the organism to adapt and/or repair the damage (49). Cells have evolved complex genetic systems to detect specific forms of stress and activate the expression of genes whose products increase the resistance of the cell to further stress and/or initiate the processes of tissue regeneration. Unfortunately, the expression of many of these genes is attenuated in aging (53, 63). As a consequence, cellular responsiveness to stress diminishes with advancing age.

One of the best understood cellular responses to stress has been traditionally called the heat shock response. Cell stresses including heat stress and exercise result in preferential transcription and translation of heat shock proteins (HSPs). Overexpression of 72-kDa HSP (HSP72) is associated with protection of cardiomyocytes from a variety of stresses including myocardial ischemia-reperfusion (I/R) injury (33, 50). Heat stress-induced increases in myocardial HSP72 are associated with reduced myocardial damage after I/R in young animals (12, 13). Furthermore, we and others (14, 23, 24, 43, 45, 56, 60) have shown that endurance exercise elevates myocardial HSP72 and protects against myocardial I/R injury in young adult animals.

Evidence indicates that mammalian aging is associated with decreased cellular expression of HSP72 in response to heat stress (6–8, 16, 21, 22, 26, 27, 38, 40–42). At present, the mechanism to explain the age-related decline in myocardial HSP72 expression is unknown. One proposed mechanism is that aging results in decreased activation of heat shock transcription factor (HSF)-1, decreased binding of HSF-1 to the heat shock element (HSE), and blunted transcriptional competency due to age-related defects at one or more stages of the multistep pathway of regulation (10, 11). Whether senescence results in a blunted response to exercise stress is currently unknown. Importantly, it appears that exercise may represent a unique stress involving protective mechanisms different than those associated with heat stress. Kregel and Moseley (34), for example, reported an increase in HSP72 in the liver of senescent animals after exercise stress but not after heat stress. The mechanism(s) responsible for this unique effect of exercise was not investigated. Furthermore, the differential effects of exercise and heat stress on myocardial HSPs with aging have not been investi-

gated. Therefore, the objective of this study was to investigate the effect of senescence on exercise-induced expression of myocardial HSP72. On the basis of preliminary experiments in our laboratory, we hypothesized that exercise-induced increases in myocardial HSP72 are diminished in old animals and that the mechanism responsible for this age-related impairment in cardiac HSP72 expression is not due to impaired HSF-1 activation.

METHODS

Animals and experimental design. This project was approved by the University of Florida Institutional Animal Care and Use Committee and followed the guidelines for animal use established by the American Physiological Society. The Fischer-344 rat was chosen as a model of aging because *1)* this animal has a relatively short life span with senescence attained at 24 mo, *2)* extensive background data are available, *3)* this stock and strain has been characterized under well-defined environmental and genetic conditions with respect to age-associated changes, and *4)* old rats are available from a reliable commercial breeder who maintains a barrier-reared aging colony of Fischer-344 rats under the close supervision of the National Institute of Aging. Young adult (6 mo) and aged (24 mo) male Fischer-344 rats, obtained from the National Institute of Aging, were individually housed, maintained on a 12:12-h light-dark cycle, provided food and water ad libitum, and randomly assigned to one of the following experimental groups: *1)* young adult control (n = 6), *2)* aged control (n = 6), *3)* young adult exercise trained (n = 8), *4)* aged exercise trained (n = 8), *5)* young adult heat stress (n = 8), and *6)* aged heat stress (n = 8).

Exercise training protocol. Animals assigned to exercise-trained groups were habituated to treadmill running over a 5-day period. During this time, both treadmill speed and running time were increased so that by *day 4*, animals were able to run continuously for 60 min at ~70% maximal oxygen comsumption ($\dot{V}O_{2\,max}$) (Table 1). Animals performed 2 consecutive days of exercise training at an intensity of ~70% $\dot{V}O_{2\,max}$, which corresponds to running speeds of 25 m/min in aged rats and 30 m/min in young adult rats on a 0% incline (37). To account for the stress of handling, control animals were placed on a nonmoving treadmill daily.

Hyperthermia protocol. Because whole body hyperthermia has been shown to result in binding of HSF-1 to the HSE and in induction of both HSP72 mRNA and protein, heat stress was incorporated into the experimental design as a positive control to compare with exercise training. Animals subjected to heat stress were anesthetized with pentobarbital sodium (35 mg/kg ip) and placed on a heating pad set at 45°C. The animals remained on the heating pad until their rectal temperature reached 41.2°C. This temperature was maintained for 15 min. As the animal recovered from anesthesia, 10–15 ml water was administered orally. A temperature of 41.2°C was chosen because it was tolerated well by the aged rats and because it resulted in increased myocardial HSP72 (44).

Table 1. *Summary of daily exercise training duration*

Exercise Groups	Training Duration, min				
	Day 1	*Day 2*	*Day 3*	*Day 4*	*Day 5*
Group 3 (5–6 mo old)	30	40	50	60	60
Group 4 (23–24 mo old)	30	40	50	60	60

Tissue removal and preparation. Animals were euthanized with an intraperitoneal injection of pentobarbital sodium (90 mg/kg), and hearts were quickly removed and rinsed free of blood. The left ventricle was separated into sections, frozen in liquid nitrogen, and stored at -80°C until assay. Death occurred within 60 min of exercise or heat stress for measurement of HSF-1 activation and Northern blot analyses or 24 h after exercise or heat stress for Western blot analyses. Rats with documented pathology at the time of death were not included in the data analysis.

Portions of the left ventricle were homogenized in 5 volumes of extraction buffer (25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM HEPES, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonylfluoride; pH 8.0) (43, 52). Homogenates were centrifuged at 15,000 *g* for 20 min. Protein concentration of the supernatant was estimated using the Bradford technique (9).

Western blotting. The transcriptional activation factor HSF-1, the constitutive isoform HSP73, and the inducible isoform HSP72 were analyzed in left ventricular samples using standard Western blotting methods described elsewhere (14, 23). Briefly, protein extracts from control, heat-stressed, and exercised rat hearts were mixed with Laemmli sample buffer, heat denatured, separated on a 12% polyacrylamide gel, and transferred to nitrocellulose membranes (35). Membranes were incubated with the primary monoclonal antibodies (Stressgen, Victoria, British Columbia, Canada) to HSP72 (SPA-810) and HSP73 (SPA-815) or the polyclonal antibody against HSF-1 (SPA-901), followed by incubation with appropriate secondary antibodies. Quantification was performed using computerized densitometry. Average intensities are expressed as a percentage of control young adult values.

Electromobility shift assay. Protein extracts (50 μg) from control, heat-stressed, and exercised rat hearts were incubated with a ^{32}P-labeled self-complementary HSE oligonucleotide (5'-TCTAGAAGCTTCTAGAAGCTTCT-3') in binding buffer (10% glycerol, 50 mM NaCl, 1.0 mM EDTA, 20 mM Tris, 1.0 mM DTT, and 0.3 mg/ml BSA; pH 8.0) with 0.065 ng (50,000 counts/min) of ^{32}P-labeled oligonucleotide and 5.0 μg poly (dIdC) for 20 min at room temperature (43). To determine the specificity of binding, reaction lysates were incubated in the presence of competing unlabeled HSE (10- or 100-fold excess). Incubation reactions were separated on a 4.5% nondenaturing polyacrylamide gel. Gels were dried and exposed to film overnight.

RNA isolation and Northern blot analyses. Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform technique, and 10 μg of total RNA were separated on a 1% formaldehyde agarose gel. After transfer to a nylon membrane and being fixed by baking at 80°C, blots were prehybridized at 42°C in 5x Denhardt's solution, 5x saline-sodium citrate (SSC; 1x SSC is 0.15 M NaCl and 0.015 M sodium citrate; pH 7.0), 50 mM K_2HPO_4 (pH 7.0), and 50 μg/ml denatured salmon sperm DNA for 4 h at 42°C. Blots were then probed with a 1.7-kb *Eco*RI fragment of the human HSP70 gene labeled with [^{32}P]CTP using the random prime method (17). Hybridization was carried out for 12 h at 42°C in 5x Denhardt's solution, 5x SSC, 10% dextran sulfate, and 50 μg/ml denatured salmon sperm DNA. Blots were washed in 0.1% SDS and 0.1x SSC before autoradiography overnight at -80°C. After being stripped, blots were reprobed with a [^{32}P]ATP end-labeled 24-bp oligonucleotide for a fragment of 28S rRNA to control for loading (5).

Statistical analysis. Comparisons between experimental groups (young adult vs. aged) for each dependent variable were made by factorial ANOVA. When indicated, a Tukey's

post hoc test was performed. Significance was established at $P < 0.05$.

RESULTS

Morphometric characteristics. Mean (±SE) body mass and heart weights of the animals for both age groups are presented in Fig. 1. Within the same age group, body mass, heart weight, and heart weight-to-body mass ratios did not differ among the experimental groups. However, compared with young adult rats, aged animals demonstrated a greater body mass and heart weight ($P < 0.05$).

Western blot analyses. Figure 2 illustrates typical Western blots to determine myocardial HSP72, HSP73, and HSF-1 levels in the control, heat-stressed, and exercise-trained groups from young adult and aged animals. Control animals from young adult and aged groups expressed similar basal levels of myocardial HSP72. Both heat stress and exercise training resulted in a significant induction of HSP72 in the myocardium of both young adult and aged rats ($P < 0.05$). This increase was significantly greater with exercise training compared with heat stress regardless of age ($P < 0.05$). Compared with young adult animals, aged animals expressed significantly less myocardial HSP72 after heat stress and exercise ($P < 0.05$). Control animals from both young adult and aged groups expressed similar levels of HSF-1 and HSP73. Neither heat stress nor exercise training increased myocardial levels of HSF-1 or HSP73 in young adult or aged animals ($P > 0.05$).

Electromobility shift assays. Figure 3 illustrates HSF-1-HSE binding in control, heat-stressed, and exercise-trained groups from both young adult and aged animals. Myocardial extracts from both young adult and aged control rats revealed negligible or absent HSF-1-HSE binding. After both exercise and heat stress, HSF-1-HSE binding was detected in myocardial extracts from both young adult and aged animals. Although we observed a diminished level of myocardial HSP72 protein expression in aged animals after both heat stress and exercise, HSF-1-HSE binding did not differ between young adult and aged animals after either stress.

mRNA analyses. Compared with unstressed controls, both exercise and heat stress resulted in increased mRNA as measured by Northern blot analyses (Fig. 4). Heat stress resulted in a greater amount of mRNA compared with exercise training. However, no differences existed between the young and aged animals.

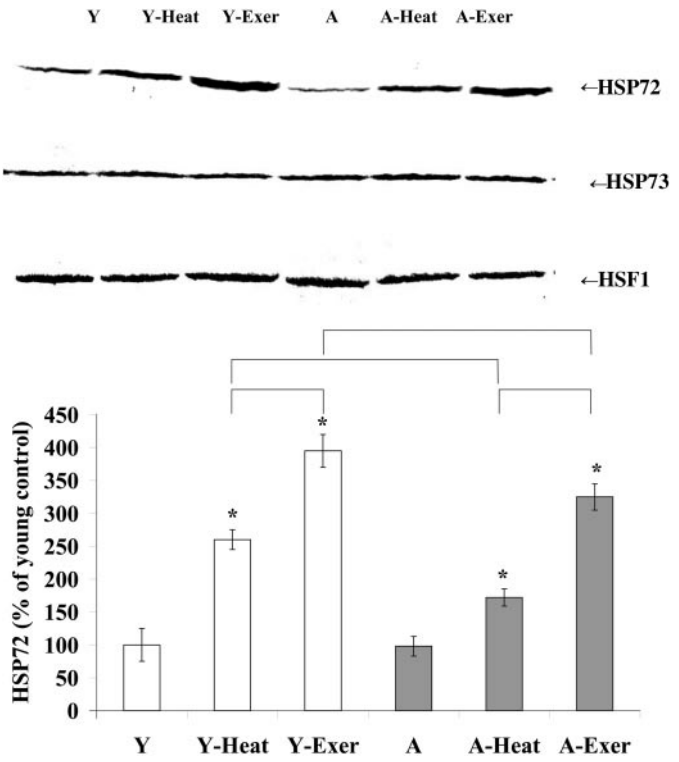

Fig. 2. Representative Western blots for heat shock protein (HSP)72, HSP73, and heat shock factor (HSF)-1. Heat, heat stress; Exer, exercise. *Significantly different from both young adult (Y) and aged (A) controls ($P < 0.05$). Further significant differences ($P < 0.05$) are indicated by brackets.

DISCUSSION

These experiments tested the hypothesis that exercise-induced increases in myocardial HSP72 are diminished in old animals and that the mechanism responsible for this age-related impairment in cardiac HSP72 expression is not due to impaired HSF-1 activation. Our data clearly support this postulate. To our knowledge, this is the first study to demonstrate that aging is also associated with diminished myocardial HSP72 induction in response to exercise stress. This is signif-

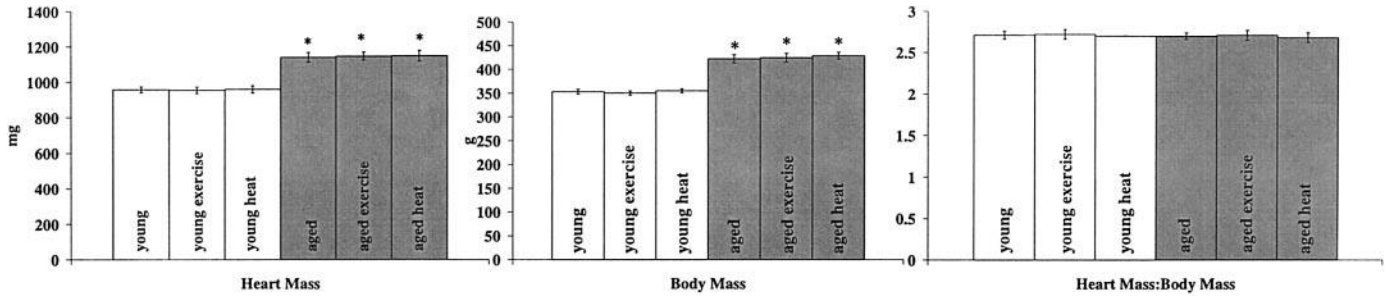

Fig. 1. Morphometric characteristics for all experimental groups. *Significantly different from all young adult groups ($P < 0.05$).

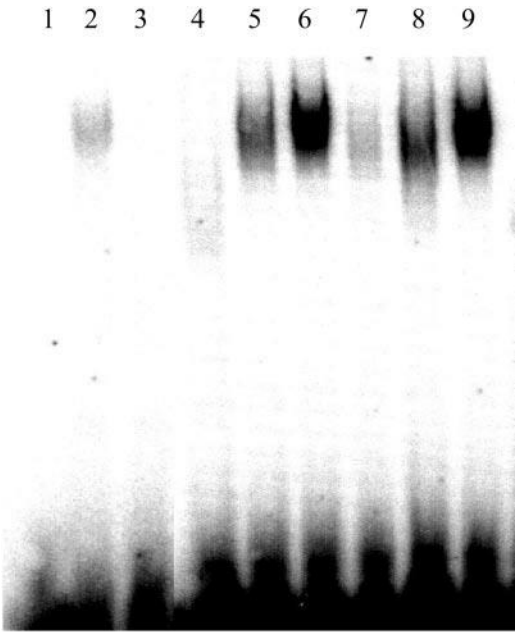

Fig. 3. Electromobility shift assay. Lane assignments are shown at the *bottom*. All treatment groups show positive binding of HSF-1 to the HSE consensus sequence.

icant given the role that HSP72 plays in providing cellular protection against a variety of stresses, including myocardial I/R injury (12, 13, 29, 32, 48, 54, 55, 57, 62). This blunted stress response may explain, at least in part, the increased susceptibility of the aged myocardium to acute stresses such as I/R injury (2, 4, 30, 39, 44, 59). While several mechanisms could contribute to this increased susceptibility to cellular injury (i.e., changes in glycogen content, norepinephrine release, protein kinase C translocation, etc.), a decreased ability to express HSP72 may also play a role. Indeed, it has been suggested that a minimum level of HSP72 is required to facilitate cardioprotection and that this protection is lost when cellular levels of HSP72 are below this critical level (44). Hence, this possibility highlights the importance of improving our understanding of those factors responsible for the age-related attenuation of myocardial expression of HSP72. In the following paragraphs, we will discuss the potential mechanisms of age-related attenuation of exercise-induced myocardial HSP72 expression as they relate to the findings of the present study.

Attenuation of the cellular stress response could be the result of one or more independent mechanisms. Decreased presence of the transcriptional activator HSF-1 in aged cells is one potential cause. However, our data indicate that myocardial HSF-1 levels do not differ between young and old animals. This finding agrees with a previous report (28) indicating that no differences in HSF-1 levels exist between young adult and senescent hepatocytes. Decreased HSF-1 activation and HSE binding is another potential cause of the attenuated stress response in senescent animals. Indeed, decreased binding of HSF-1 to the HSE has been observed in hepatocytes isolated from old rats (25), aging human fibroblasts (42, 46), and myocardium from whole body heat-stressed aged rats (44). This observation could be due to repression of HSF-1 trimerization, a critical step in the acquisition of transcriptional competency. HSF-1 trimerization may be repressed via recruitment of HSF-binding protein-1, a complex of HSPs that induces dissociation of HSF-1 oligomers (11). Changes in pH, phosphorylation status, temperature, and redox environment can also impact the oligomerization of HSF-1 monomers (61). The results of the present study, however, revealed no differences in HSF-1 activation and-HSE binding after either heat stress or exercise in young versus aged heart tissue. Hence, in the present study, the mechanism responsible for attenuated exercise-induced HSP72 expression in aged animals does not appear to be associated with HSF-1 availability, oligomerization, or HSE binding. Furthermore, a diminished exercise stress response might also be the result from alteration in the final modulation of HSF-1 (i.e., phosphorylation of HSF) leading to transcriptional competence. To investigate this possibility, we measured the presence of HSP72 mRNA after exercise and heat stress. Our results indicate that myocardial HSP72 mRNA levels do not differ between young and aged animals following heat stress or exercise. The observation that HSP72 mRNA was greater after heat than after exercise seems to be further proof that there is not a detriment in the capacity to make mRNA in response to exercise. Therefore, the age-related attenuation of exercise-induced expression of myocardial HSP72 is not due to the failure to acquire transcriptional competency.

Collectively, our data reveal that low myocardial levels of HSF-1, impaired HSF-1 activation, or the failure to acquire transcriptional competency cannot

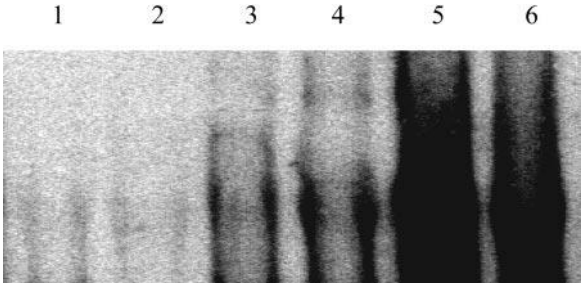

Fig. 4. mRNA analysis. Lane assignments for this representative Northern blot are as follows: *lane 1*, aged control; *lane 2*, young adult control; *lane 3*, aged exercise; *lane 4*, young adult exercise; *lane 5*, aged heat; and *lane 6*, young adult heat.

explain the age-related attenuation of exercise-induced expression of myocardial HSP72. Hence, by elimination, we postulate that the depressed expression of myocardial HSP72 in old animals after exercise is due to other molecular events such as decreased mRNA stability, impaired translation resulting in reduced synthesis of the HSP72 protein, and/or a decreased half-life of HSP72 protein. The current data cannot define which of these potential explanations is responsible for the age-related decrease in myocardial HSP72 expression after exercise. Nonetheless, a brief discussion of each of these potential mechanisms is warranted. First, preferential degradation of mRNA containing AU-rich elements has been described (36, 61). In this regard, HSP72 mRNA contains a 3'-untranslated region AU-rich element that could serve as a tag for rapid degradation by proteolytic pathways such as the ubiquitin-proteosome pathway (36). Unfortunately, it is currently unknown whether HSP72 mRNA is more rapidly degraded in old animals compared with young adults. This is an interesting area for future research.

Furthermore, whether accelerated errors of translation, changes in rates of translation, or changes in posttranslational modification of HSP72 are associated with aging remain largely unstudied. Dukan et al. (15) proposed that aging may be associated with an increase in translational errors and have demonstrated that mistranslated proteins are more susceptible to oxidation. These authors speculate that oxidation, in the form of irreversible carbonylation, destines these aberrant proteins for degradation rather than for repair/refolding (15). Finally, while the incidence of translation errors may increase in aged cells leading to damaged proteins and accelerated oxidation, there is growing evidence that protein turnover decreases with aging (19, 20, 47, 58). Specifically, experimental evidence indicates age-related declines in the activities of both lysosomal and proteasomal protein degradation pathways (19, 20, 47, 58). Hence, it seems unlikely that the age-related attenuation of HSP72 is the result of a decreased half-life of HSP72 protein.

Another important point relevant to these experiments is the possible age-related difference in cellular responses to exercise stress compared with other stresses (i.e., heat) traditionally employed to elicit a stress response. Exercise has long been considered a noninvasive and potentially valuable intervention to offset age-related physiological changes in a variety of cells (18). While some of the cellular changes that result from exercise stress appear to parallel those observed with other stresses such as heat shock, it is possible that exercise serves as a unique trigger of cellular responses. For example, in the present study, heat stress resulted in greater myocardial levels of HSP72 mRNA compared with exercise in both young and old animals. Nonetheless, compared with heat stress, exercise resulted in a greater accumulation of HSP72 protein in the hearts of both young and old animals. This observation suggests a differential effect of heat stress versus exercise on RNA stability, translation, and/or protein stability. Further support for this notion can be found in a 1993 study (51) reporting evidence that the 3'-UTR of HSP70 is, in fact, heat responsive. As mentioned previously, it has been reported that the heat shock response is preserved in senescence after an exertional hyperthermic stressor compared with passive hyperthermia (34). Other metabolic changes resulting from exercise stress and shown to elicit changes in expression of stress proteins include energy depletion, pH disturbances, production of reactive oxygen species, and possibly protein damage. Precisely how these cellular disturbances interact to elicit changes that render cells more resistant to subsequent stresses remains undefined. Recent evidence suggests that exercise is associated with preservation of an otherwise blunted protection associated with ischemic preconditioning in aged hearts (1, 3). Interestingly, Abete et al. (1, 3) have reported that exercise restores the protection afforded by ischemic preconditioning in both an animal model of aging as well as in humans with preinfarction angina, the clinical counterpart to ischemic preconditioning. Clearly, additional studies are needed to elucidate the unique cellular changes associated with exercise and how these changes might preserve the cardioprotective effects of interventions such as ischemic preconditioning and hyperthermia during senescence.

In summary, our results demonstrate for the first time that aging is associated with diminished myocardial HSP72 induction in response to exercise stress and that this diminution is not due to HSF-1 activation or the acquisition of transcriptional competency. While it remains unclear whether an attenuation of the cellular stress response is a cause versus a consequence of aging, cellular resistance to aging has been associated with longevity (31), which provides undeniable support for the notion that the stress response is important in aging. Because molecular chaperones such as HSP72 are ubiquitous and participate in such a wide variety of cellular processes, it is probable that the manifestations resulting from decrements in cellular expression of HSPs are far reaching (47). It is also noteworthy that cellular tolerance to stress cannot be attributed exclusively to HSP overexpression. Indeed, many other mechanisms are involved, including expression and regulation of antioxidant enzymes, modulation of proteolytic pathways and DNA repair proteins, and modifications of phospholipid bilayer composition (for a review, see Ref. 61). Age-associated regulation of stress-response genes is an area of accelerated research, particularly with the advent of microarray technology (63). The information resulting from such research, in addition to the advances in the area of gene therapy, will be valuable in defining the roles of HSPs and other components of the stress response in aging.

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DISCLOSURES

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