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Myocardial Heat Shock Protein 70 Expression in Young and Old Rats After Identical Exercise Programs

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Synthesis of inducible heat shock protein 70 (HSP70) is impaired in aged animals following acute stresses including exercise. In this study we determined whether aging affects expression of this cytoprotective protein following chronic exercise participation. Male Fischer 344 rats, final ages 6 and 24 months, exercised identically for 10 weeks on a treadmill (15° incline, 15 m/min for up to 60 minutes, 5 days/week). In 6-month-old animals, exercise increased HSP70 in heart (44%), liver (216%), and skeletal muscle (126%) ($p < .05$ vs sedentary). In 24-month-old animals, exercise increased HSP70 in muscle (69%), but not in heart or liver. In heart, antioxidant enzyme activities and HSP70 messenger RNA were measured and found to be unaffected by exercise at both ages. Our results indicate an age-related decrease in HSP70 production in heart and liver following chronic exercise. Furthermore, the aged heart does not increase its antioxidant enzyme defenses to compensate for the HSP70 deficit.

EXERCISE and brief heat stress are among a group of relatively mild stresses capable of inducing intrinsic adaptations that provide protection against a variety of subsequent, more severe stresses (1–4 for reviews). Although most cell types are affected, the heart has received particular attention because of the therapeutic importance of maintaining the health of this organ. A key aspect of the adaptive process by the heart is de novo synthesis of protective proteins; however, there is uncertainty regarding the specific proteins involved (1–4). Proteins reported to have prominent roles in exercise-induced cardioprotection include inducible heat shock protein 70 (HSP70), superoxide dismutase (SOD), and catalase (5–10). The antioxidant enzymes function to prevent damage to proteins and other cellular components during oxidative stress associated with hyperthermia, exercise, or ischemia, whereas HSP70 aids in the subsequent recovery by promoting restoration of dysfunctional enzymes and preventing aggregation of severely denatured proteins (11–13 for reviews). Specific cardioprotective functions attributed to HSP70 include repairing ion channels, protecting mitochondrial function, inhibiting proinflammatory cytokines, and attenuating apoptotic cell death by suppressing the c-Jun NH_2-terminal kinase signaling pathway (11–14). Together, the protective mechanisms of HSP70 and antioxidants are considered important for a strong defense against cellular stresses.

Several studies conclude that HSP70 induction is impaired in the heart of aged animals following a variety of acute stresses including ischemia (15), heat (16–20), and exercise (20,21). An age-related decline in the HSP70 response has also been observed in a variety of other tissues including liver and skeletal muscle (22–24). Although there is a general consensus that aging results in a decline in the induction of HSP70, the molecular mechanism remains unresolved. Investigations carried out in heat-stressed liver suggest that decreased binding ability of heat shock transcription factor (HSF1) to the heat shock element within DNA plays a role in that tissue (22); however, Demirel and colleagues (20) reported no such decline in hearts of aged rats after either heat or exercise stress. Most of what we know about HSP expression and aging comes from observations following acute stresses; very few studies have used stresses on a chronic basis. In one chronic study, Naito and colleagues (25) found that young and aged rats increased skeletal muscle HSP70 by similar amounts after 10 weeks of daily (5 days/week) exercise. This finding suggests that cardiac muscle of aged animals may also be capable of increasing HSP70 to levels comparable to those in young animals in response to chronic exercise training, but need a longer time due to the considerable age-related decline in overall protein synthesis (26). Such a scenario is consistent with the classic study by Florini and colleagues (27), who showed that a similar amount of cardiac hypertrophy could occur in both young and old rat hearts, but the process takes longer in the old animals.

Alternatively, other protective proteins, such as antioxidant enzymes, may increase in the aged heart following chronic exercise to compensate for low HSP70 expression. Consistent with this hypothesis, two studies that measured myocardial antioxidant enzymes in 21-month-old Fischer 344 rats after chronic exercise have reported exercise-induced increases in MnSOD (28) and catalase (28,29). However, whether such an adaptation represents compensation for lack of HSP70 production or is specific to aged hearts is not known because HSP70 was not measured and younger animals were not included in the studies. We recently found that a single, acute exercise stress produces different protective phenotypes in young (4 month)

compared to old (21 month) rat hearts (21). Specifically, the increase in HSP70 24 hours postexercise was drastically blunted in the old animals, but MnSOD increased by 45% in the old group and did not change in the young group. Catalase displayed a similar percentage increase at both ages, but the old group had higher activity both before and after exercise. Importantly, both age groups displayed similar improvements in tolerance to an ischemia–reperfusion stress administered 24 hours postexercise. It remains to be determined whether the antioxidant enzymes and HSP70 would continue to differ in the young and old animals as they adapt to a chronic exercise program. Thus, the primary purpose of this study was to determine the effect of age on changes in myocardial HSP70 and antioxidant enzymes following a chronic (10–12 week) exercise program. HSP70 expression was also measured in liver and skeletal muscle, two organs that have also received considerable attention regarding age-related changes in the HSP response.

Methods

Animals and Exercise Protocol

Young (3 months) and old (22 months) male Fischer 344 rats were obtained from the aged rodent colony maintained by the National Institute on Aging. The animals were randomly divided into four groups: young sedentary ($n = 10$), young exercise-trained ($n = 10$), old sedentary ($n = 10$), and old exercise-trained ($n = 10$). After a 3-day habituation to a motor-driven treadmill consisting of walking at 5 m/min on a level surface for 5 min/day, exercisers of both age groups began a 5 day/week running program at a speed of 15 m/min up a 15° incline. During the first 3 weeks the exercise duration was gradually increased from 10 to 60 min/day in all animals. The protocol was then maintained at this duration for the remaining 7–9 weeks of the 10- to 12-week exercise program. At the completion of the exercise program, animals were anesthetized with sodium pentobarbital (60 mg/kg, I.P.) and killed by decapitation, and the tissues were dissected free. The heart, medial vastus lateralis muscle, and portion of liver were quickly wrapped in aluminum foil and stored at −80°C until analyzed as described below. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 85-23, revised 1996).

HSP70 Expression

Inducible HSP70 was identified by western blotting and quantified by densitometry as summarized below and described in detail previously (7). Approximately 150 mg of tissue was homogenized in 20 volumes of HEPES buffer (25 mM N-2-hydroxyethypiperazine-N′-2-ethanesufonic acid, 1 mM EDTA, pH 7.4) with a Teflon-glass Potter–Elvehjem homogenizer immersed in ice. Protein concentration in the homogenate was determined according to the method of Lowry, and an aliquot was diluted 1:1 with Laemmli sample buffer (125 mM Tris [pH 6.8], 20% v/v glycerol, 2% w/v sodium dodecyl sulfate, 0.008% w/v bromophenol blue, and 200 mM dithiothreitol). Aliquots containing 80 μg of total protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% resolving mini-gels in duplicate. The proteins were then transferred to a polyvinylidene difluoride (PVDF) sheet and blotted with HSP70 mouse monoclonal immunoglobulin G (sc-24; Santa Cruz Biotechnology, Santa Cruz, CA). Then the membranes were blotted with sheep anti-mouse immunoglobulin, horseradish peroxidase-linked whole antibody (NXA931; Amersham, Piscataway, NJ) and detected with SuperSignal chemiluminescent substrate luminol/enhancer (Pierce, Rockford, IL). The resulting labeled bands were quantified using an image scanner and an image analysis software program (NIH Image 1.66; Bethesda, MD). On each gel, a standard sample, created from the myocardium of a heat-shocked rat, was loaded along with the samples of the treatment groups. The concentration of HSP70 in the standard sample and the experimental samples was determined to be within the linear range of detection. The HSP70 content of each sample was reported as a percentage of the standard loaded on each gel and was adjusted for the concentration of protein loaded for the respective sample.

HSP70 Messenger RNA

The amount of HSP70 messenger RNA (mRNA) was determined using a quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) procedure and normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA as a relative control standard. Total RNA was isolated from a portion of the left ventricle with TRI Reagent RNA Isolation Reagent (Sigma Chemical Co., St. Louis, MO) using a modification of the Chomczynski and Sacchi RNA isolation method (30). Purity of total RNA was estimated by the A260/A280 ratio, and the minimum acceptable ratio was set at 1.7. RNA was then reverse transcribed using Oligo(dT)15 as described in the ImProm-II Reverse Transcription System kit (Promega, Madison, WI), and the complementary DNA (cDNA) was amplified using the PCR Core System 1 (Promega). The HSP70 sense primer sequence was 5′TCGAGGAGGTGGATTAGAG3′, and the antisense sequence was 5′GGGATGCAAGGAAAAAAC3′ (31). The GAPDH sense sequence was 5′GGTGAAGGTC GGTGTCAACGGATT3′, and the antisense sequence was 5′GATGCCAAAGTTGTCATGGATGACC3′ (32). The HSP70 amplification reaction (50 μl final volume) contained 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 U of Taq polymerase, 3 μg of cDNA, and 50 pmol of HSP70 or 100 pmol of GADPH sense and antisense primers. The GAPDH amplification reaction (50 μl final volume) contained 2.0 mM $MgCl_2$, 200 μM each dNTP, 1 U of Taq polymerase, 3 μg of cDNA, and 100 pmol of GADPH sense and antisense primers. The amplifications were carried out in a Bio-Rad Gene Cycler thermal cycler (Hercules, CA). The amplification profile for HSP70 was 35 cycles consisting of 30 seconds at 94°C, 1 minute at 57°C, and 1 minute at 72°C. The amplification profile for GAPDH was 20 cycles consisting of 30 seconds at 94°C, 1 minute at 59.5°C, and 1 minute at 70°C. Concentrations of RNA, mRNA, and DNA were quantified spectrophotometrically at 260 nm. PCR products were analyzed on 2% agarose gels containing ethidium bromide.

Table 1. Animal Characteristics

Group	Body Weight (g)	Heart Weight (mg)	Heart Weight/Body Weight (mg/g)	Vastus Medialis Cytochrome Oxidase (μmoles O_2/min/g wet wt)
6-mo SED	400 ± 9 (10)	949 ± 15 (10)	2.38 ± 0.03 (10)	34.1 ± 1.6 (6)
6-mo RUN	382 ± 7 (9)	960 ± 21 (9)	2.52 ± 0.04* (9)	50.3 ± 1.9* (6)
24-mo SED	402 ± 20 (10)	1041 ± 31 (10)	2.65 ± 0.14† (10)	30.9 ± 3.9 (6)
24-mo RUN	369 ± 9 (10)	1019 ± 38 (10)	2.76 ± 0.08† (10)	52.2 ± 3.2* (6)

Notes: Values are means ± standard error for number of animals in parentheses.
*$p < .05$ vs SED.
†$p < .05$ vs 6 mo.
SED = sedentary; RUN = runner.

Antioxidant Enzymes

Left ventricular tissue was homogenized with a Teflon-glass homogenizer in 20 volumes of 50 mM KH_2PO_4, 0.1 mM EDTA, 0.1% (v/v) Triton X-100, pH 7.4. After low-speed centrifugation at 1500 *g* for 10 minutes, the supernatant was analyzed for antioxidant enzyme activity. Catalase activity was measured polarographically using a Clark-type oxygen electrode according to del Rio and colleagues (33) on the same day the tissue was homogenized. The remaining supernatant was frozen at –80°C for later determinations of SOD and glutathione peroxidase (GPX). Preliminary determinations indicated that the activities of SOD and GPX are not affected by a single freeze–thaw cycle. Manganese and copper–zinc SOD, representing the mitochondrial and cytosolic forms respectively, were measured by spectrophotometric analysis at 550 nm according to McCord and Fridovitch (34), and GPX was measured at 340 nm according to Gunzler and Flohé (35). All tissue-preparation steps were carried out at ice-cold temperatures, and enzyme assays were run at 25°C.

Cytochrome Oxidase

Cytochrome oxidase was selected as the marker of skeletal muscle mitochondria content, which is known to increase in proportion to intensity of an endurance training protocol (36). Whole medial lateralis muscle was cut into small pieces then homogenized with a Teflon-glass homogenizer in 20 volumes of 50 mM KH_2PO_4, 0.1 mM EDTA, 0.1% (v/v) Triton X-100, pH 7.4. Cytochrome oxidase activity was then determined polarographically at 24°C as previously described (37).

Statistical Analysis

All data are expressed as means ± standard error. Differences among groups were analyzed using a two-way analysis of variance with post hoc analysis utilizing Tukey's Honestly Significant Difference (HSD) test. The alpha level for statistical significance was set at 0.05.

Results

Body and Heart Weights

Body weights, heart weights, and heart weight-to-body weight ratios are summarized in Table 1. An increase in heart mass of 9.7% occurred in the sedentary animals from the age of 6–24 months ($p < .05$), but body weight did not differ. The exercise program did not change heart weight or body weight compared to sedentary controls at either age. However, there was a modest increase in heart weight-to-body weight ratio in the young exercise group ($p < .05$) due to nonsignificant trends toward decreased body weight and increased heart weight.

Cytochrome Oxidase

Exercise training increased cytochrome oxidase activity in the vastus medialis muscle by 48% in the young animals and 69% in the old (Table 1). Similar increases in mitochondria content occurred in the gastrocnemius muscle and are reported in a companion study (38). The magnitude of the increases indicates that the exercise program provided an effective training response, especially in the old animals.

HSP70 Expression

Figure 1 displays HSP70 protein content in left ventricle, liver, and vastus medialis muscle of sedentary and

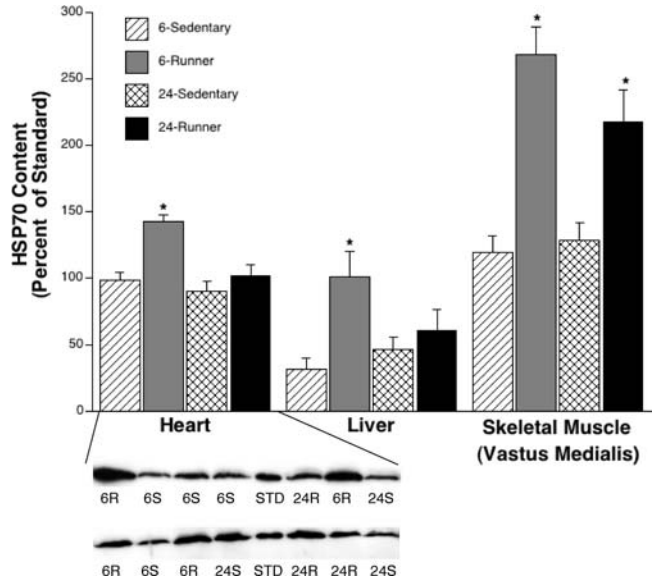

Figure 1. Heat shock protein 70 (HSP70) content in left ventricle, liver, and vastus medialis muscle relative to a standardized HSP70 solution. Values are means ± standard error. Scan of western blots from the left ventricle of 3–4 animals in each group is immediately below the histogram. *Significantly different from sedentary at same age ($p < .05$). S = sedentary; R = runner; STD = HSP70 standard.

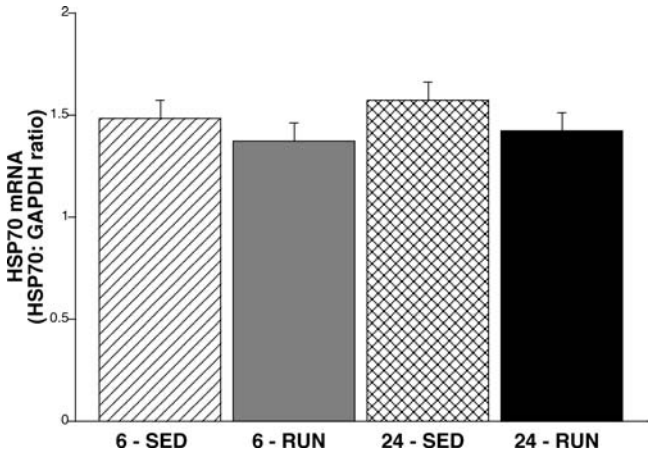

Figure 2. Heat shock protein 70 (HSP70) messenger RNA (mRNA) content in left ventricle normalized to glyceraldyhe-3-phosphate dehydrogenase (GAPDH) mRNA. Values are means ± standard error. No differences were observed at the 0.05 level of significance.

exercise-trained rats. No significant age-related differences were observed for levels of inducible HSP70 in any tissue from sedentary animals. The chronic exercise stimulus induced increases ($p < .05$) in vastus medialis HSP70 content in both young (125%) and old (70%) animals. The young runners also responded with significant ($p < .05$) increases of HSP70 in the left ventricle (45%) and liver (233%). However, HSP70 content did not increase in either of these organs in the old runners ($p > .05$). Figure 2 shows that left ventricle HSP70 mRNA levels were unchanged by exercise or by aging.

Antioxidant Enzymes

Activities of catalase, SOD, and GPX in the left ventricle are reported in Table 2. The exercise program did not alter any of the antioxidant enzymes in either young or old animals. Catalase activity increased 50% from the age of 6–24 months ($p < .05$), but the other antioxidant enzymes were not affected by aging.

Table 2. Antioxidant Enzyme Activity

Enzyme	6-Month SED	6-Month RUN	24-Month SED	24-Month RUN
Catalase	1414 ± 162	1275 ± 52	2097 ± 127*	1949 ± 115*
SOD total	2656 ± 140	2636 ± 156	2991 ± 157	2872 ± 219
CuZnSOD	1188 ± 68	1244 ± 172	1329 ± 202	1168 ± 151
MnSOD	1468 ± 169	1392 ± 195	1662 ± 186	1704 ± 159
GPX	468 ± 20	450 ± 33	447 ± 31	447 ± 33

Notes: Values are mean Units/g wet weight ± standard error for $N = 9–10$. Catalase Unit = 1 μmole H_2O_2/minute; SOD Unit = 50% inhibition of cytochrome *c* reduction; GPX Unit = 1 μmole glutathione/minute.

*$p < .05$ vs 6 mo.

SED = sedentary; RUN = runner; SOD = superoxide dismutase; GPX = glutathione peroxidase.

Discussion

It has been widely reported that the ability to increase the expression of myocardial HSP70, a key cytoprotective protein, is attenuated in aged animals. Most of these studies attempted to elevate HSP70 expression with a single hyperthermic stress (16–20,22,23) or acute exercise (20,21). If the attenuation were due simply to a decrease in the rate of synthesis, conceivably after several repeated stresses the protein would eventually increase to a level commensurate with the intensity of the stress. The present study clearly indicates that after 3 months of exercise for 5 days/week HSP70 expression is not increased in heart or liver of aged rats, although it is significantly elevated in young rats (Figure 1). The lack of increase in the aged animals is not due to lower exercise intensity by this group, as they were exercising at the same absolute intensity, duration, and frequency as were the younger animals, and displayed similar increases in skeletal muscle mitochondria content. In fact, because the old animals would be expected to have a lower aerobic capacity, they were likely exercising at a greater relative intensity than the young animals were.

Although HSP70 expression did not increase in heart or liver, it increased in the vastus medialis muscle of the old rats after the exercise program to a level only slightly below that of the young runners (Figure 1). This observation is in agreement with the finding of Naito and colleagues (25) who measured HSP70 expression in several skeletal muscles (but no other tissues) of 6-month-old and 26-month-old rats after a 10-week program of treadmill exercise. They found that HSP70 increased similarly in some skeletal muscles from both young and old rats, and was only slightly attenuated with age in others. The combined implication from the present study and that of Naito and colleagues is that age-related decrements in the HSP response to chronic exercise are tissue specific.

There are several potential explanations for the attenuated HSP70 induction in the aged animals, and the mechanism may differ by organ and/or type of stress imposed. For example, decrements at the level of HSF1 are considered important in liver and heart following whole-body heat stress (19,22), but are reportedly not involved in the attenuated HSP70 response in the heart following exercise (20). We observed that myocardial HSP70 mRNA levels were similar in young and aged animals (Figure 2); this observation supports the findings of Demirel and colleagues (20) that HSF1 availability, oligomerization, heat shock element binding, or overall transcriptional competence is not the cause of low myocardial HSP70 levels after exercise. To be consistent with other studies evaluating the impact of chronic exercise on gene expression, we measured mRNA levels 24 hours postexercise to avoid acute effects from the most recent exercise bout (39–41). Therefore, we cannot rule out the possibility that decrements in transcription during the first few hours after each exercise bout are involved. However, this seems unlikely, because the aged hearts had greater HSP70 mRNA relative to protein than did the younger hearts. Thus our results appear consistent with the overall conclusion of Demirel and colleagues, that mechanisms other than dysfunction at the level of HSF1 activation and transcription of mRNA are responsible for the impaired HSP70 induction in the aged heart.

We hypothesized that old Fischer 344 rats would respond to chronic exercise by increasing myocardial MnSOD and catalase to compensate for the decrement in HSP70 production. This hypothesis was based on our previous finding that these enzymes increased in previously sedentary old rats 24 hours after a strenuous exercise bout (21) and findings of Kim and colleagues (29) and Somani and Rybak (28), who found elevations of myocardial antioxidant enzymes following chronic running in old Fischer 344 rats. Our hypothesis appears to be false, because the chronic exercise program did not affect the activity of any antioxidant enzyme in the heart of old or of young rats (Table 2). These findings are consistent with several other chronic exercise studies using young rats of various strains (7,42–46) or old Wistar rats (47,48), but they appear to conflict with those of Kim and colleagues and Somani and Rybak. A possible explanation for the apparent conflict is that the antioxidant status of the sedentary animals in the studies by Kim and colleagues (29) and Somani and Rybak (28) may have been depressed compared to younger animals and the exercise programs returned some of the antioxidant enzymes to normal levels, i.e., prevented age-related decreases. This was not necessary in our study because no age-related declines in antioxidant enzymes occurred (see discussion of age-related changes below). What is the explanation for why myocardial MnSOD and catalase were increased 24 hours after a single strenuous exercise bout by old rats in our previous study (21), but not elevated after 3 months of daily exercise in the present study? The explanation is based on the fact that a single exercise bout by a previously sedentary animal is more stressful than a similar exercise bout by an animal that has adapted to exercise over several months. Some studies supporting this explanation include the following. Somani and colleagues (49) found that an acute bout of exercise resulted in greater increases of myocardial MnSOD and catalase in previously sedentary rats compared to chronically trained rats even though the acute exercise was more intense for the trained animals. Boluyt and colleagues (50) found greater increases in myocardial c-Jun NH_2-terminal kinase after exercise in previously sedentary rats compared to their chronically exercised counterparts. Also, in a study investigating the time course of adaptation to a chronic, moderate exercise program, Harris and Starnes (7) observed that changes in antioxidant enzymes occurred during the early phase of the program but returned to sedentary values over time as the animals adapted to the exercise. Studies that have reported increases in myocardial SOD of young rats following a chronic exercise program used programs that were more intense than those that were used herein (51,52).

Although no exercise-induced changes in antioxidant enzymes were apparent, there was an age-related increase of 50% in myocardial catalase activity, whereas SOD and GPX were unaffected by age (Table 2). Considerable controversy exists regarding the effect of aging on myocardial antioxidant enzymes. Even when only the Fischer 344 rat is considered, reported age-related effects of myocardial antioxidant enzymes vary considerably. In fact, for all of the enzymes measured herein, studies can be found reporting increases, decreases, and no change in this rat strain (21,53–61). The reason for such divergent results among different laboratories is not apparent; however, our results herein are consistent with our previous findings in regard to both aging (21) and chronic exercise training (7,46). Age-related upregulation of cardiac antioxidant enzymes has been proposed as a means to counter age-related increases in oxidant exposure and maintain oxidant homeostasis (59,62), and at least one study (62) has concluded that an increase of catalase is the key adaptation to counter mitochondrial H_2O_2 production in aged rat hearts. Perhaps the upregulation of catalase by the rats of the present study is adequate to counter age-related increases in oxidant production, and no further adaptation is necessary when moderate exercise is added to their lifestyle. Clearly, more research is necessary to understand the cellular defense strategies of the aging heart.

Summary

We found that when young and old rats participate in identical chronic exercise programs, the old rats do not increase myocardial HSP70 or antioxidant enzyme content. We focused exclusively on these proteins because the vast majority of reports on exercise-induced cardioprotection point to them as key mediators in the protective adaptation that occurs in young rats (5–10,21,51,52). Although aging clearly attenuates the exercise-induced expression of HSP70, we cannot rule out the possibility that it could increase to therapeutic levels in heart and liver in response to a more intense exercise program. Also, at least one investigator has reported improved cardioprotection following acute (but not chronic) exercise without increases in either HSP70 or any of the antioxidant enzymes measured herein (63). Thus it appears that other cytoprotective proteins may also be induced by exercise, but the exact nature of other potential protective proteins or strategies is as yet unclear. Finally, although the exercise program used herein did not increase selected myocardial proteins, it is important to point out that low-to-moderate intensity exercise is known to provide other adaptations that provide cardioprotective benefits. Regular exercise participation at a minimum intensity of 40%–60% maximum oxygen consumption is sufficient to result in decreased sympathetic drive to the heart, lower blood pressure, improved lipid profile, and decreased insulin resistance (64). Lowered heart rate and systolic blood pressure during submaximal activities reduces myocardial work, thereby providing a direct anti-ischemic effect by reducing myocardial oxygen demands and coronary blood flow requirements. Thus, low-to-moderate intensity exercise does result in cardioprotective benefits, and the present study helps clarify the mechanisms involved in aged animals.

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