Exercise training inducibility of MnSOD protein expression and activity is retained while reducing prooxidant signaling in the heart of senescent rats

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Lawler JM, Kwak HB, Kim JH, Suk M. Exercise training inducibility of MnSOD protein expression and activity is retained while reducing prooxidant signaling in the heart of senescent rats. Am J Physiol Regul Integr Comp Physiol 296: R1496–R1502, 2009.—While the stress response to heat and exercise is limited in the heart with progressive aging, recent data indicate that acute or short-term exercise upregulates the Mn isoform of superoxide dismutase (MnSOD), which may provide protection against ischemia-reperfusion injury and cell death by reducing oxidative stress. Growing evidence indicates that inducible nitric oxide synthase (iNOS) contributes to age-induced increases in oxidative stress and risk of heart failure. We postulated that oxidative stress and iNOS levels would be related to the ability of the aging heart to upregulate MnSOD in response to long-term exercise training. Six- and twenty-seven-mo-old Fischer-344 rats had been assigned to young sedentary (YS), young exercise (YE), old sedentary (OS), or old exercise (OE) groups. ET groups ran on a treadmill for 60 min/day, 5 days/wk for a total of 12 wk. MnSOD protein expression in the left ventricle was increased (+43%) by 12 wk of exercise training in the old age group, with no changes in Cu,ZnSOD. Exercise training also increased MnSOD activity in left ventricles from old and young rats. HSP70 was inducible by exercise training in hearts exclusively from the young age group. iNOS protein expression increased markedly with aging (+548%), while exercise training decreased iNOS levels by −73% in OS compared with YS. In addition, 4-hydroxynonenal protein adducts in the left ventricle increased by 237% with aging, while 12 wk of exercise training resulted in attenuation (−55%). These data indicate that inducibility of MnSOD is preserved with long-term exercise training in the aging rat heart. Moreover, upregulation of MnSOD in the aging heart was directly associated with attenuated levels of oxidative stress, including iNOS.

aging; heart; oxidative stress; superoxide dismutase; inducible nitric oxide synthase

AGING INCREASES SUSCEPTIBILITY of the heart to oxidative stress, ischemia-reperfusion injury, and a reduction in cardiac contractility (e.g., stroke volume and ejection fraction) (2, 20, 21, 25, 38, 49,61). Impaired cardiac contractility is believed to be a function of impaired Ca2+ homeostasis and progressive mechanical remodeling of the myocardium (49, 51). Mechanical remodeling observed in the aging heart, particularly in males, is characterized by loss of myocytes, symptomatic of accelerated apoptosis and necrosis along with reduced replacement by adult stem cells (3, 12, 51). In addition, the remaining myocytes undergo significant reactive hypertrophy (3, 33, 38, 56). Moreover, connective tissue and fibrosis of the heart increase with aging, altering geometry and mechanical function of the myocardium (33, 38, 56).

Putative upstream regulators of apoptosis, necrosis, and remodeling include 1) increased oxidative and nitrosative stress and 2) impaired response of stress response proteins, including mitochondrial Mn-dependent isoform of superoxide dismutase (MnSOD), heat shock proteins (HSPs), and IGF-1 (16, 44, 47, 51, 52). The mitochondrial Bcl-2 pathway and activation of caspase-3 play a significant role in age-associated increases in apoptosis (21). Potential sources of increased oxidative stress in the aging heart include elevated mitochondrial leakage of reactive oxygen species (ROS) and increased levels of inducible nitric oxide synthase, or iNOS (5, 6, 8, 15, 26, 28). Indeed, pharmacological inhibition or knockout of iNOS protected against impairment of baseline contractility, ischemia-induced contractile dysfunction, and elevated caspase-3 activation in the aging heart (26, 35). Previously, high levels of reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite (ONOO−), have been shown to inhibit MnSOD and Cu,ZnSOD activities, usually via nitration (24, 53).

If exercise training does indeed reduce apoptosis, myocyte loss, and remodeling in the aging heart, then it is possible that it may do so by increasing stress response proteins, such as MnSOD and HSP70, and subsequent reduction of oxidative stress (21). Recently, we demonstrated that exercise training attenuated age-induced elevation in Bax/Bcl-2 ratio, caspase-9, caspase-3, and apoptosis in the rat heart (21). Leeuwenburgh and colleagues also reported that lifelong wheel running reduced H2O2 mitochondrial production in isolated mitochondria in hearts from old rats (16). Our laboratory was the first to demonstrate that exercise training increases superoxide dismutase activity and reduces oxidative stress in hearts from young rats (36). A recent study indicates that acute or a limited number of days of exercise promote an upregulation of MnSOD in the aging heart as well (11).

However, data examining the inducibility of MnSOD with long-term aging studies are sparse. This is significant as MnSOD is a mitochondrial antioxidant enzyme that attenuates apoptosis via modulating the mitochondrial Bcl-2 pathway through reduction in Bax and upregulation of Bcl-2 (1, 21, 32, 43, 51). In addition, recent intriguing data suggest exercise may modulate iNOS and MnSOD though a common pathway (14). Alternatively, HSP70 upregulation may result in a reduction in iNOS levels (59).

Therefore, we hypothesized that upregulation of MnSOD in response to long-term exercise training would be retained in the aging heart. We further postulated that upregulation of MnSOD as a result of 12 wk of treadmill exercise training would be linked to reduced oxidative stress and lower levels of iNOS in the left ventricles of old Fischer-344 rats.
Exercise training upregulates MnSOD in old rats

METHODS

Animals. We purchased 3- and 24-month-old Fischer-344 rats from the NIA colony as our young adult and old groups, at the initiation of our study. Animals were cared for at the Comparative Biology facility at Texas A&M University in accordance with National Institutes of Health and the University Laboratory Animal Care Committee standards. An animal use protocol had been approved by Texas A&M University to cover all procedures in the study. Rats were housed in a temperature-controlled (23 ± 2°C) room and kept on 12:12-light-dark diurnal cycle. Water and rat chow were provided ad libitum. One-half of the rats in each age group ran for 12 wk (5 days/wk) on a motorized treadmill for 60 min/day, and the other half served as sedentary age-matched controls: young sedentary controls (YS) (n = 10), young exercise trained (YE) (n = 10), old sedentary controls (OS) (n = 10), and old exercise trained (OE) (n = 10). After 12 wk of exercise training, the young and old rats in each age group were euthanized. At the time of death, sedentary and exercise rats were 6 and 27 mo of age for the young and old age groups.

Exercise training protocol. Rats in the exercise groups ran on a motor-driven treadmill at 15 m/min up a 15° incline, 1 h/day, 5 days/wk for 12 wk. Rats in the exercise groups were gradually conditioned to perform this level of exercise over the first 5 wk of the 12-wk training program. The intensity was designed to correspond to 75% of VO2 max in the old group (23). The first 5 days will be an acclimation period for rats to adapt to the treadmill machine without incline at 15 m/min for 10 min. Rats will be gradually conditioned to perform the exercise on a 15° incline up to an hour over the first 3 wk of the 12-wk training program. This exercise regimen has previously been shown to increase heart/body mass ratio and elevate citrate synthase activity, a marker of oxidative capacity (21). Heart weight/body weight ratio and citrate synthase activity have been assessed as an indicator of the efficacy of the exercise training regimen and have been consistently upregulated using similar protocols (21, 45).

Tissue preparation. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip) 48 h following the last exercise training bout in young and old age groups to reduce the effect of the last exercise bout. The left ventricle was quickly extracted, weighed, and placed in ice-cold PBS (pH = 7.4). We focused on the left ventricle since it is a primary target of age-induced remodeling in the heart (18). The left ventricle samples were frozen in liquid nitrogen and stored at −80°C until analysis.

Left ventricle samples were weighed, minced into fine pieces, and placed in ice-cold (4°C) lysis buffer (pH = 7.5) containing the following: 20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% Igepal CA630, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 0.1 mM DTT, and protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany) at 4°C. Tissues samples were homogenized (20 vol/wt) in lysis buffer using a ground glass on ground glass homogenizer (Bellco Biotechnology, Vineland, NJ), and then centrifuged twice at 10,000 g (4°C) for 10 min to remove particulate matter. Sample protein levels were determined using an Endogen kit.

Superoxide dismutase activity was measured as described previously (24) with MnSOD activity assessed using 1 mM KCN to inhibit Cu,Zn SOD activity. 0.02 U/ml xanthine oxidase, 6 mM xanthine, and 60 μM cytochrome c are mixed with 300 μl of homogenate for a final volume of 1 ml. The change in absorbance at 550 nm was subtracted from a control using 300 μl phosphate buffer, with 1 U of SOD defined as a 50% reduction in Δ absorbance.

Western immunoblotting. Protein expression for MnSOD, Cu,ZnSOD, HSP70, iNOS, and 4-hydroxynonenal adducts was determined by Western immunoblot analysis (21). Separating gel (375 mM Tris·HCl; pH = 8.8; 0.4% SDS; 10% acrylamide monomer) and stacking gel (125 mM Tris·HCl; pH = 6.8; 0.4% SDS; 10% acrylamide monomer) solutions were made. Polymerization of acrylamide was initiated by tetramethylethylenediamine and ammonium persulfate. Separating and stacking gels were quickly poured into a Bio-Rad Protein III gel-box (Bio-Rad, Hercules, CA). Thirty micrograms of protein from heart homogenates in sample buffer (Tris pH = 6.8 with 2% SDS, 30 mM DTT, 25% glycerol) were loaded into the wells of the 10% polyacrylamide gels, and electrophoresed at 150 V. The gels were then transferred at 30 V overnight onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were then blocked in 5% nonfat milk in PBS with 0.1% Tween-20 at room temperature for 6 h. After being washed 3 times in PBS with 0.4% Tween-20, membranes were incubated at room temperature with appropriate primary antibodies for MnSOD (1:10, 000; Santa Cruz Biotechnology, Santa Cruz, CA), Cu,ZnSOD (1:200; Transduction Laboratories, Lexington KY), HSP70 (1:5,000; Stressgen, San Diego, CA), iNOS (1:1,000; BD Biosciences, Franklin Lakes, NJ), and 4-hydroxynonenal (1:1,333; Calbiochem, San Diego, CA) in PBS for 12 h. Following three washings in PBS and 0.4% Tween-20, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) in PBS at room temperature for 90 min. Following 3 washes in PBS with 0.4% Tween-20, an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) was used for visualization. An equal amount of protein was loaded in each well (30 μg) based on prior analysis of sample protein concentrations. To confirm equal loading and transfer consistency of muscle protein, Ponceau-S-staining was performed for each membrane after transfer to check for equal loading. As an additional control, membranes were stripped, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein levels were assessed. Moreover, lane background readings were subtracted from each lane’s protein blot density reading. Blots were then quantified as the net pixel density, subtracted from background, multiplied by area, and then equated per microgram protein.

Statistics. Two-way ANOVAs (age × exercise) with Student-Newman-Keuls as a post hoc test were used to assess group mean differences in MnSOD, Cu,ZnSOD, HSP70, iNOS, 4-hydroxynonenal, MnSOD activity, and total hydroperoxides. The level of significance was set at P < 0.05.

RESULTS

Heart mass/body mass ratio and gastrocnemius citrate synthase levels were increased by exercise training in both age groups (cited in Ref. 21), confirming the efficacy of the exercise training regiment employed.

Protein expression for MnSOD in the left ventricle as a result of age and exercise training is displayed in Fig. 1A. Protein levels for MnSOD trended downward (−7%) but did not reach statistical significance (P = 0.10). However, exercise training resulted in upregulation of MnSOD protein expression in both young (+29%) and old (+43%) age groups. In contrast with changes in MnSOD, Cu,ZnSOD protein expression levels were assessed. Moreover, lane background readings were subtracted from each lane’s protein blot density reading. Blots were then quantified as the net pixel density, subtracted from background, multiplied by area, and then equated per microgram protein.

To determine whether exercise resulted in an upregulation of the cytotoxic isoform of superoxide dismutase in the aging heart, we measured protein expression of Cu,ZnSOD (Fig. 2). In contrast with changes in MnSOD, Cu,ZnSOD protein expression actually trended slightly upward with age (+4%). However, the difference in means was not statistically significant (P = 0.90). Moreover, exercise had no effect on Cu,ZnSOD protein levels in either age group. Indeed, Cu,ZnSOD activity levels were also unaffected by either age or exercise (data not shown). Thus, exercise training specifically affected protein...
expression of the mitochondrial isoform of superoxide dismutase but not the cytosolic isoform in the aging rat heart.

We then determined whether the antioxidant chaperone stress protein HSP70 was inducible in the aging heart in response to 12 wk of exercise training. Exercise training elicited a modest increase (+27%) in HSP70 protein expression in left ventricles from young rats. In contrast with the pattern observed with MnSOD, we found that HSP70 protein levels did not respond to exercise training in the aging heart (Fig. 3). No age effect for HSP70 protein expression was noted as well.

As RNS and ROS produced by high levels of iNOS may contribute to oxidative stress, apoptosis, and alterations in MnSOD, we sought to determine whether exercise training attenuated age-induced upregulation of iNOS protein expression in the rat heart. Indeed, iNOS protein levels increased markedly (+548%) in the left ventricle as a function of aging (Fig. 4). Exercise training had no significant effect on iNOS protein expression in the young age group. However, there was a remarkable 73% decrease in ventricular iNOS protein levels...
in response to exercise training in left ventricles from the senescent rats.

We then tested the hypothesis that MnSOD upregulation in response to exercise training in the aging heart was not a function of high oxidative stress (14) but was associated with lower oxidative stress. 4-hydroxynonenal adducts, a marker of oxidative stress, were 237% higher in the hearts of aging rats compared with young, sedentary counterparts (Fig. 5A). Exercise training significantly reduced 4-HNE by 62% in the left ventricles in the old age group. Similarly, total hydroperoxides increased in the left ventricle with age (394%) but was decreased by exercise training in the old (−55%) and young age groups (Fig. 5B).

DISCUSSION

Our results indicate that the ability of long-term exercise training to upregulate protective MnSOD protein and activity levels is retained in the aging heart. However, exercise training had no effect on ventricular Cu,ZnSOD levels, regardless of age. Exercise training increased HSP70 protein expression in left ventricles from young rats. However, inducibility of HSP70 in response to endurance exercise training was lost in the hearts of old rats. Exercise training was effective in reducing iNOS protein levels and oxidative stress in the left ventricles in both the young and old age groups. Thus, the aging rat heart continues to exhibit an antioxidant and stress response when challenged by 12 wk of treadmill exercise training, as a function of inducibility of MnSOD, but not Cu,ZnSOD or HSP70.

MnSOD activity in the left ventricle decreased as aging progressed (Fig. 1B). However, age-induced changes in MnSOD activity were not reflective of a reduction in MnSOD protein levels. Hollander et al. (13) also noted similar decreased in MnSOD activity, but not protein levels in aging skeletal muscle. These data suggest that aging may reduce MnSOD activity and antioxidant protection in the heart through post-translational modification of MnSOD. These findings have important ramifications, as they imply reduced antioxidant protection in the aging heart, where ROS production is increased (16, 17).

Previously, superoxide dismutase was shown to be inducible in the young heart in response to chronic exercise training (37). Recent studies indicated that a single bout of exercise or 5 days of exercise resulted in upregulation of MnSOD in hearts from old rats (31, 49). Upregulation of MnSOD gene transcripts occurred with exercise in the heart of middle-aged rats (45). Karkula et al. (20) and Pushpalatha et al. (40) reported increases in total superoxide dismutase activity in response to exercise training using middle-aged rats (12- and 18-mo Wistar rats). However, protein levels were not assessed, nor were rats
used beyond their mean lifespan, and in fact considerably younger. Ji (14) had previously reported no augmentation of total superoxide dismutase activity in the aging heart with exercise training. Our data clearly indicate that the ability of exercise to upregulate mitochondrial MnSOD protein levels and activity, with no change in Cu,ZnSOD protein or activity levels, in senescent rats is maintained with extended exercise-training periods. It is important to note that rats used in the current study were 3 mo past their mean lifespan at the conclusion of the exercise-training period. In addition, it was also evident that exercise-induced elevation of MnSOD activity was related to an increase in MnSOD protein levels in the senescent heart, although post-translational modification is certainly possible.

Of particular importance is that upregulation of left ventricular MnSOD levels in the aging heart in response to exercise was indicative of a compensation against an elevation in oxidative stress. Aging has long been associated with elevated oxidative stress in the heart (14, 16, 17, 35, 37, 38, 42, 51, 58, 59). Moreover, increased mitochondrial production of ROS has been consistently measured in the aging heart as well (16). Exercise-induced reduction of oxidative stress in the heart has been previously described by a number of investigators (16, 38, 40). In the current study, oxidative stress levels were significantly lower as a result of exercise training in the aging heart. Therefore, upregulation of MnSOD protein levels and activity observed in the aging heart with exercise training (Fig. 1) are consistent with MnSOD’s role as a mitochondrial antioxidant in the aging heart. Indeed, Venditti et al. (56) first described reductions in mitochondrial H$_2$O$_2$ leakage after exercise training in skeletal muscle. Starnes et al. (47) recently reported that exercise training reduced mitochondrial ROS leakage, using a similar exercise regimen. In addition, Judge et al. (16) also found that lifelong wheel running decreased mitochondrial H$_2$O$_2$ leakage in aging heart and skeletal muscle. Our findings imply that exercise training reduces oxidative stress in the aging left ventricle, at least in part, by increasing the mitochondrial antioxidant enzyme MnSOD.

Previous data consistently have demonstrated that the major antioxidant enzyme MnSOD is most consistently upregulated by exercise training (12, 37, 52). In contrast, catalase and glutathione peroxidase are typically nonresponsive to exercise training in the heart (52). Our Cu,ZnSOD data are consistent with a negligible responsiveness of the cytosolic Cu,Zn isoform of superoxide dismutase to exercise training in hearts from both young and old rats (52). MnSOD upregulation as a result of exercise training may also play a protective role against oxidative stress, apoptosis, and heart damage that occur as a result of ischemia-reperfusion injury (41). However, MnSOD does not appear requisite for protection against ischemia-reperfusion injury. In addition, Wu et al. (59) demonstrated that overexpression of a mitochondrial-specific catalase gene, reduces oxidative stress and augments protection against cardiomyocyte contractile dysfunction. Recently, we demonstrated that exercise training reduces apoptosis, myocyte loss, and mitochondrial Bcl-2 pathway signaling in the aging heart (23). Thus, we propose that MnSOD upregulation with exercise training may play a protective role against progressive apoptosis and remodeling that can occur in the aging heart.

HSP70 may play a role in exercise protection against oxidative stress and ischemia-reperfusion injury in the heart (38, 41), although it is not an absolute requirement for I/R protection (41). While exercise stimulates increased levels of HSP70 in the heart of young rats, Starnes et al. (48) found that inducibility of HSP70 protein expression appears to be lost in response to exercise training in the aging heart. In contrast, inducibility of HSP70 in aging skeletal muscle was retained (48). Consistently, we observed that 12 wk of exercise training increased HSP70 protein expression in young hearts, but this ability was not evident in the aging heart. Therefore, it appears unlikely that protection against age-induced susceptibility to apoptosis, remodeling, and I/R injury in the heart (22, 41) with exercise training is conferred by HSP70.

Upregulation of MnSOD with exercise training in the aging heart is, therefore, particularly important, as the transcriptional response to oxidative stress and cell protection is impaired in the aging heart (9). The homozygous knockout of MnSOD dies within days of birth due to heart failure, while Van Remmen et al. (55) showed heterozygous knockdown of MnSOD results in a decline of mitochondrial function and increased MtDNA damage. Furthermore, in aging skeletal muscle, MnSOD+/− results in more oxidative stress and rapid aging (28). MnSOD overexpression may reduce oxidative stress and age-induced apoptosis of the heart (22). Therefore, it is logical that upregulation of MnSOD by exercise in the aging heart could be protective of myocytes against oxidative stress and apoptosis.

Age-induced upregulation of iNOS can contribute to oxidative stress and cardiac contractile dysfunction (27, 36, 61). Elevated iNOS levels have been linked with hypertension and chronic heart failure as well (7, 61). Although still uncertain, upregulation of iNOS has been proposed as a causative or at least secondary effect of the aging process and increased inflammatory signaling (6, 30). iNOS inhibition may protect against ischemia-reperfusion injury, caspase-3 activation, and apoptosis in the heart (27, 36). In a series of experiments Ji and colleagues (16) provided evidence that a bout of acute strenuous exercise upregulates MnSOD and iNOS as a response to oxidative stress mediated by NF-κB activation in skeletal muscle. Our data indicate a divergence with chronic exercise between expression of MnSOD and iNOS, where MnSOD signaling is maintained, while iNOS is downregulated. Thus, the “homesis hypothesis” may be more appropriate for a single bout of exercise, consistent with a “preconditioning” role. Further study is warranted. It is possible that the ability of exercise training to reduce iNOS protein expression in the aging heart could be a potential mechanism for protection conferred by long-term exercise training against age-related apoptosis and remodeling (23). However, no cause and effect have been established.

A reduction in iNOS and upregulation of MnSOD could also attenuate production of peroxynitrite by reducing availability of superoxide anion and nitric oxide (25, 32). Previously, we demonstrated that RNS inactivate superoxide dismutase activity, including MnSOD (25). For example, increased peroxynitrite downregulated MnSOD from muscle and heart (25). Gielen et al. (10) found that exercise training reduced iNOS and protein nitrosylation, while improving mitochondrial cytochrome oxidase activity in skeletal muscle of heart failure patients. We found that age-induced changes in MnSOD activity in the left ventricle were not simply a product of protein expression, implying posttranslational modifications or protection that enhanced enzymatic activity (Fig. 1). Therefore, it is
possible that exercise training may enhance MnSOD activity in the aging heart by reducing ROS and RNS, thus contributing to myocyte protection against age-induced apoptosis and cardiac remodeling (22, 23). Future research is warranted to determine whether exercise-induced reduction in iNOS in the aging rat heart plays a permissive role in MnSOD augmentation, or is downstream of the effects of exercise on MnSOD protein expression and activity levels.

Perspectives and Significance

Inducibility of MnSOD in the heart in response to exercise training was retained in the aging heart. Upregulation of MnSOD with exercise was not in response to elevated oxidative stress but was related to lower oxidative stress and iNOS expression. In contrast, other stress response proteins, specifically Cu,ZnSOD and HSP70, were not responsive to exercise training in the aging heart. These data are consistent with the hypothesis that the ability to elevate protein and activity levels of MnSOD in the heart is conserved by aging, and may serve as a mechanism of cardiac protection against oxidative stress, as well as age-induced remodeling.

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R1502 EXERCISE TRAINING UPREGULATES MNSOD IN OLD RATS


