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Exercise by lifelong voluntary wheel running reduces subsarcolemmal and interfibrillar mitochondrial hydrogen peroxide production in the heart

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Judge, Sharon, Young Mok Jang, Anthony Smith, Colin Selman, Tracey Phillips, John R. Speakman, Tory Hagen, and Christiaan Leeuwenburgh. Exercise by lifelong voluntary wheel running reduces subsarcolemmal and interfibrillar mitochondrial hydrogen peroxide production in the heart. *Am J Physiol Regul Integr Comp Physiol* 289: R1564–R1572, 2005. First published July 28, 2005; doi:10.1152/ajpregu.00396.2005.—Evidence suggests that mitochondrial dysfunction and oxidant production, in association with an accumulation of oxidative damage, contribute to the aging process. Regular physical activity can delay the onset of morbidity, increase mean lifespan, and reduce the risk of developing several pathological states. No studies have examined age-related changes in oxidant production and oxidative stress in both subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria in combination with lifelong exercise. Therefore, we investigated whether long-term voluntary wheel running in Fischer 344 rats altered hydrogen peroxide (H_2O_2) production, antioxidant defenses, and oxidative damage in cardiac SSM and IFM. At 10–11 wk of age, rats were randomly assigned to one of two groups: sedentary and 8% food restriction (sedentary; $n = 20$) or wheel running and 8% food restriction (runners; $n = 20$); rats were killed at 24 mo of age. After the age of 6 mo, running activity was maintained at an average of 1,145 ± 248 m/day. Daily energy expenditure determined by doubly labeled water technique showed that runners expended on average ~70% more energy per day than the sedentary rats. Long-term voluntary wheel running significantly reduced H_2O_2 production from both SSM (−10.0%) and IFM (−9.6%) and increased daily energy expenditure (kJ/day) significantly in runners compared with sedentary controls. Additionally, MnSOD activity was significantly lowered in SSM and IFM from wheel runners, which may reflect a reduction in mitochondrial superoxide production. Activities of the other major antioxidant enzymes (glutathione peroxidase and catalase) and glutathione levels were not altered by wheel running. Despite the reduction in mitochondrial oxidant production, no significant differences in oxidative stress levels (4-hydroxy-2-nonenal-modified proteins, protein carbonyls, and malondialdehyde) were detected between the two groups. The health benefits of chronic exercise may be, at least partially, due to a reduction in mitochondrial oxidant production; however, we could not detect a significant reduction in several selected parameters of oxidative stress.

aging; superoxide anion; longevity; postmitotic; calorie restriction

AGING IS ASSOCIATED WITH AN increase in mitochondrial DNA deletions (5, 14, 21) and a decline in mitochondrial function (12, 13, 16), leading to increased mitochondrial oxidant production (38, 39, 42, 56, 57). Furthermore, oxidative damage to DNA, lipids, and proteins has been shown to increase as a function of age (6, 58). Regular physical activity may have a benefit on mitochondrial function, but the precise mechanisms by which exercise exerts its effects have not been determined. However, it has been hypothesized that the health benefits of chronic exercise may be at least partially due to a reduction in oxidant production and oxidative damage. In rodents, exercise training (forced treadmill running or swimming) has been shown to reduce the basal rate of mitochondrial hydrogen peroxide (H_2O_2) production (64) and to enhance antioxidant enzyme activity (32, 35, 48, 49, 55), adaptations that would be expected to reduce tissue oxidative damage. Indeed, oxidative damage to DNA, proteins, and lipids has been reported to be lower in tissues from exercise-trained animals compared with sedentary animals (51–53, 63). However, forced modes of exercise can cause physiological adaptations indicative of chronic stress (37).

An alternative to forced exercise paradigms (e.g., treadmill running or swimming) is to allow subjects free access to running wheels, thus allowing individuals to exercise voluntarily for extended periods of time. Voluntary wheel running increases mean lifespan (23, 24), and survival is significantly enhanced in wheel-running rats compared with treadmill-trained rats (40). There is also evidence that exercise may reduce oxidative damage and increase antioxidant enzyme activities in a variety of tissues (29, 30, 33); more recently, it has been shown that long-term voluntary exercise can attenuate many age-related changes in gene expression (inflammatory response, stress response, signal transduction, and energy metabolism) (10). However, little is known regarding the specific effects of long-term voluntary wheel running on mitochondrial

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function and oxidative stress in the heart. Moreover, cardiac muscle contains two distinct populations of mitochondria: subsarcolemmal mitochondria (SSM), which are located beneath the plasma membrane, and interfibrillar mitochondria (IFM), which are found in parallel rows between the myofibrils (16). State 3 respiratory rates, content of respiratory cytochromes, and activities of electron transport chain complexes are higher in IFM compared with SSM, suggesting higher metabolic demands on IFM (46). Furthermore, protein yield and maximal state 3 respiration decline in IFM with age, whereas SSM remain relatively unaffected (16).

We determined the effects of long-term (21 mo) voluntary wheel running on mitochondrial oxidant production, antioxidant enzyme activities, and oxidative damage in SSM, IFM, and cytosol isolated from hearts of 24-mo-old rats and also investigated whether SSM and IFM were differently affected by voluntary wheel running. We found that H_2O_2 production from both SSM and IFM was reduced in the exercised animals. However, this reduction in oxidant production was not associated with reduced levels of measured oxidative stress biomarkers.

EXPERIMENTAL PROCEDURES

Animals. Male Fischer 344 rats were purchased from Harlan (Indianapolis, IN) at 10–11 wk of age and were housed at the University of Florida (Gainesville, FL) until killed at 24 mo of age. One week after arriving at our facilities, rats were randomly assigned to one of two groups: sedentary with 8% food restriction (sedentary; $n = 20$) or wheel running with 8% food restriction (runners; $n = 20$). Rats fed ad libitum tend to abruptly decrease their running activity, and slight food restriction (8–10%) has been shown to prevent this decline (25, 26). Food intake for both groups of rats (sedentary and runners) was therefore restricted by 8% below the ad libitum food intake of a separate group of sedentary, age-matched, male Fischer 344 rats. Throughout the duration of the study, food intake of these two groups was adjusted accordingly each week (based on ad libitum food intake from the previous week). All animals were singly housed in a temperature- (20 ± 2.5°C) and light-controlled (12:12-h light-dark cycle) environment with unrestricted access to water. All sedentary rats were housed in standard rodent cages supplied by the University of Florida's Animal Care Services. Rats in the wheel-running group were housed in cages equipped with Nalgene activity wheels (1.081 m circumference) obtained from Fisher Scientific (Pittsburgh, PA) and had free access to the wheels. Each wheel was equipped with a magnetic switch and an LCD counter that recorded the number of wheel revolutions. The number of revolutions was recorded for each animal daily. Body weights of all rats were recorded weekly. At the time of death (24 mo), there were 12 in each group. All experimental procedures were approved by the University of Florida's Institute on Animal Care and Use Committee.

Daily energy expenditure. Daily energy expenditure (DEE) was estimated by using the doubly labeled water technique as previously described (59, 60) in sedentary and wheel-running rats at 10 mo of age under license at the University of Florida. In summary, rats were weighed, and then an intraperitoneal injection of deuterium (enrichment = 4.63 atom%) and ^{18}oxygen (enrichment = 9.44 atom%) was administered. The syringes containing the isotope were weighed to 0.0001 g pre- and postinjection to obtain the dose mass. After isotope injection, the rats were returned to their original cages (with access to food and water) for 1 h to allow isotope equilibration with the animal's water pool. After the 1-h equilibration period, an initial blood sample was collected by tail tipping (50-μl Vitrex precalibrated capillary syringes). All blood samples were immediately flame sealed and stored until analyses. Final blood samples were collected exactly as above, 24 h after the initial sample was taken. In addition, background samples were collected to determine the naturally occurring enrichments of deuterium and ^{18}oxygen. Analysis of samples was carried out at the University of Aberdeen, UK, using mass spectrometry and following methods previously described (59, 60). Results were expressed as kilojoules per day.

Mitochondrial isolation. Animals were euthanized with isoflurane (administered via inhalation using a precision vaporizer at 5%), and the hearts were perfused with PBS, removed, rinsed in saline, blotted dry, and weighed. SSM and IFM were isolated according to the method of Palmer et al. (47) with slight modification (16). After trimming excess fat and removing the atria, we minced and homogenized the ventricles 1:10 (wt/vol) in ice-cold Chappell-Perry buffer (100 mM KCl, 50 mM MOPS, 1 mM EDTA, 5 mM $MgSO_4 \cdot 7H_2O$, 1 mM ATP, pH 7.4). The homogenates were then centrifuged at 500 *g* for 10 min at 4°C (all remaining centrifugation steps were also performed at 4°C). The supernatant was transferred to a new tube, and the pellet was resuspended and centrifuged at 500 *g* for 10 min. The two supernatants were subsequently pooled and centrifuged at 3,000 *g* for 10 min to obtain SSM, whereas the pellet obtained in the second low-speed centrifugation step was saved for IFM isolation. After the first 3,000-*g* spin, the supernatant (crude cytosolic fraction) was aliquoted and stored at −80°C, whereas the pellet was resuspended and centrifuged at 3,000 *g* for 10 min. This wash step was repeated once more, and the final SSM pellet was suspended in 1 ml of Chappell-Perry buffer and kept on ice until use. Meanwhile, nagarse (5 mg/g wet wt tissue) was added to the tissue pellet containing IFM, incubated on ice for 5 min, and homogenized. The homogenate was then diluted twofold with Chappell-Perry buffer and centrifuged at 5,000 *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in the original volume of buffer. The resuspended pellet was centrifuged at 500 *g* for 10 min to yield the nuclear pellet. The supernatant was saved; to improve recovery of IFM from the nuclear pellet, the pellet was resuspended and centrifuged at 500 *g* for 10 min. This step was repeated once more with the supernatant being saved after each spin. The supernatants from the three low-speed spins were combined and centrifuged at 3,000 *g* for 10 min to obtain the IFM pellet. The IFM pellet was washed twice, and the final pellet was suspended in 0.75 ml of Chappell-Perry buffer. Protein concentrations of SSM and IFM were determined using the Bradford method (9), and mitochondrial oxygen consumption and H_2O_2 production were measured in both fractions immediately thereafter.

Oxygen consumption. Mitochondrial respiratory function was measured using a biological oxygen monitor system (model YSI 5300; Yellow Spring Instruments, Columbus, OH). Mitochondrial respiration was monitored at 37°C in incubation buffer (145 mM KCl, 30 mM HEPES, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1% fatty-acid-free albumin, pH 7.4), 2.5 mM pyruvate, 2.5 mM malate, and 0.5–1 mg mitochondrial protein for a total volume of 500 ml. State 4 respiration (no ADP) was read for 2 min and monitored by use of a chart recorder. State 3 respiration (with ADP) was then measured in the presence of 500 mM ADP for 10 min or until the oxygen pressure was equal to zero. Oxygen consumption was reported as nanomoles oxygen consumed per minute per milligram protein.

H_2O_2 production. H_2O_2 production was measured in intact mitochondria following the method of Barja (4). The use of fluorescent horseradish peroxidase-based assays for measuring H_2O_2 is well accepted in the literature (3, 4, 20, 28, 31). However, it remains possible that changes in P_{O_2} may have an effect on the total amount of H_2O_2 produced, but there should be no differences between the treatment groups. Briefly, incubation buffer (145 mM KCl, 30 mM HEPES, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1% fatty-acid-free BSA, pH 7.4) was added to test tubes followed by the addition of mitochondria (0.25 mg/ml protein), horseradish peroxidase (5.7 U/ml), homovanilic acid (0.1 mM), and substrate (2.5 mM pyruvate, 2.5 mM malate), so that the total volume was equal to 1.5 ml. The tubes were incubated in a shaking water bath at 37°C for 15

min, and the reaction was stopped by placing the tubes on ice and adding 0.5 ml of stop solution (0.1 M glycine, 25 mM EDTA-NaOH, pH 12.0) to each tube. Fluorescence was measured at an excitation wavelength of 312 nm and an emission wavelength of 420 nm using a SPECTRAmax Gemini XS dual-scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA). Arbitrary fluorescence units were converted to known amounts of H_2O_2 using a glucose-glucose oxidase standard curve. All measurements were performed in duplicate, and results are expressed as nanomoles H_2O_2 produced per minute per milligram protein.

Antioxidant enzyme activity. SOD (EC 1.15.1.1) activity was assayed according to Oyanagui (45) with slight modification. One unit (U) of SOD activity was defined as the concentration of enzyme that inhibits nitrite formation from hydroxylamine in the presence of xanthine oxidase by 50%. CuZnSOD activity was determined in the cytosol, whereas MnSOD activity was determined in the mitochondria using KCN. Selenium-dependent glutathione peroxidase (GPX; EC 1.11.1.9) activity was determined at 37°C, according to Flohe and Gunzler (18), with H_2O_2 as the substrate. Glutathione reductase (GR; EC 1.8.1.7) activity was measured at 30°C, according to Carlberg and Mannervik (11). Catalase (CAT; EC 1.11.1.6) activity was measured at 25°C according to Aebi (1).

Reduced and oxidized glutathione. Reduced and oxidized glutathione (GSH and GSSG, respectively) was measured according to the methods of Jones et al. (27) and Fariss and Reed (17). Sample proteins were precipitated with 10% (wt/vol) perchloric acid containing 10 mM of the metal chelator, diethylenetriamine pentaaceticacid. Derivatization of thiols in the acid-soluble fraction with 40 mM iodoacetic acid to form *S*-carboxymethyl derivatives was followed by fluorophore conjugation of primary amines with 75 mM dansyl chloride. GSH and GSSG were separated by normal-phase HPLC using an aminopropyl column (CEL Associates, Houston, TX). Derivatives were detected using a Hitachi fluorescence detector with an excitation wavelength of 330 nm and an emission wavelength of 515 nm. Quantification was achieved relative to authentic GSH and GSSG standards, by using γ-glutamyl-glutamate as an internal control to assess completeness of derivitization.

Protein oxidation. Protein carbonyls were measured in SSM and IFM using an enzyme immunoassay (Zentech PC test, Zenith Technology, Dunedin, NZ) with slight modification. Briefly, all samples, standards, and quality controls were normalized to 1.8 mg/ml (protein concentration of the lowest sample). Next, 11 μl of each sample, standard, and control were incubated in 19 μl of dinitrophenylhydrazine for 45 min at room temperature. After derivitization with dinitrophenylhydrazine, 7.5 μl of each sample (also standards and controls) were diluted into 1 ml of enzyme immunoassay buffer. Manufacturer's instructions were then followed starting with the section entitled ELISA procedure (section 3). All samples, standards, and controls were run in triplicate.

Lipid peroxidation. For detection of 4-hydroxy-2-nonenal (HNE)-modified proteins, proteins (15 μg) were separated using 4–20% SDS-polyacrylamide gels under denaturing conditions and then transferred to nitrocellulose. The membranes were blocked in PBS-0.05% Tween 20–5% milk and incubated with rabbit anti-HNE antiserum (Alpha Diagnostic International, San Antonio, TX) at a 1:500 dilution overnight. The next morning, the membranes were washed in PBS-0.05% Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1,000 dilution for 1 h. Enhanced chemiluminescence detection reagents from Amersham (Amersham Pharmacia Biotech, Piscataway, NJ) were used to generate a chemiluminescent signal, and bands were visualized by exposing the membranes to Hyperfilm-enhanced chemiluminescence (Amersham Pharmacia Biotech). Blots were analyzed using Kodak 1D image analysis software (Eastman Kodak, Rochester, NY). Optical density was determined by calculating the net optical density (sum of the background-subtracted pixel values) of all the bands within a given lane. As an additional marker of lipid peroxidation, we also measured thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa et al. (43), with some modifications. Samples and standards were incubated with 12.5% TCA and 0.8 N hydrochloric acid and 1% 2-thiobarbituric acid at 95°C for 1 h. After a cooling step, samples and standards were spun in a microcentrifuge tube at 10,000 rpm for 10 min. The supernatant was pipetted into a microplate, and absorbance at 532 nm was determined. All samples and standards were run in duplicate, and results are expressed as micromoles per milligram protein.

Statistical analysis. An unpaired *t*-test was used to determine whether there was a significant exercise effect (sedentary vs. runners). An unpaired *t*-test was also used to detect differences between SSM and IFM isolated from the same group of animals. Significance was set at $P < 0.05$.

RESULTS

Morphological characteristics, running wheel activity, and DEE. The average distance run per day was recorded throughout the duration of the study (see Fig. 1*A*). Although peak running activity occurred at 6 mo of age (~2,500 m/day), running activity was maintained at an average of 1,145 ± 248 m/day for the remainder of the study. This is in contrast to previous studies that showed a continual decline after approximately midlife in the average distance run per day as the animals age (23, 25). At 10 mo of age, DEE was estimated in the two groups of rats. Runners exhibited a significantly higher DEE (202 ± 31 kJ/day for runners vs. 121 ± 22 kJ/day for pair-fed sedentary controls; $P < 0.05$), expending ~70% more energy per day than the sedentary rats. This activity level, in

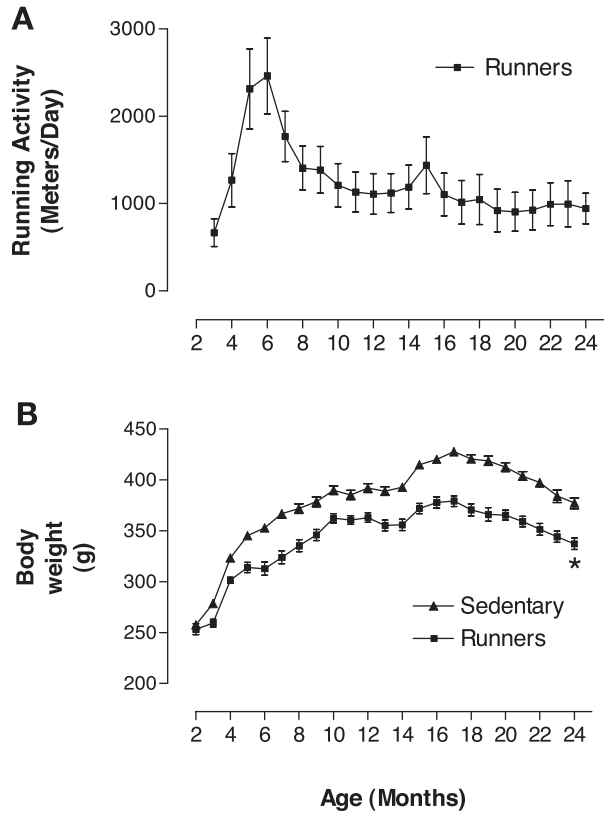

Fig. 1. *A*: average distance (meters) run per day for rats in the wheel-running group (runners). Values are means ± SE. *B*: average body weights of rats in the sedentary and wheel-running groups. Results are expressed as means ± SE. *$P < 0.05$, different at all time points in the line (except at 2 mo of age).

terms of wheel revolutions per day, remained relatively constant throughout the lifespan of the rat; therefore, potential mitochondrial adaptations were carefully examined. DEE measurements were not performed before death to avoid stress and death in the very old animals. In addition, wheel-running animals weighed significantly less than their sedentary counterparts throughout the study (Fig. 1*B*). There was no difference in heart weight between the two groups (1.04 ± 0.01 g in sedentary vs. 1.03 ± 0.02 g in runners) at the termination of the study.

Oxygen consumption. As seen in Table 1, wheel running had no effect on the rates of oxygen consumption (state 4 or state 3) or respiratory control ratio (RCR). A similar finding was reported by Servais et al. (55), who measured oxygen consumption in skeletal muscle SSM and IFM in young animals after 5 mo of voluntary wheel running. Others have reported RCR values very similar to ours (19, 31, 36). However, our oxygen consumption values and RCRs were lower than what we normally obtain. We believe this may be because we used isoflurane to anesthetize our animals in this study. Recently, isoflurane and other volatile anesthetic agents (i.e., halothane, sevoflurane) have been shown to suppress mitochondrial respiration (61). However, we chose to use isoflurane because it afforded us the ability to harvest tissue (and therefore rapidly the mitochondria) from several animals in a much shorter time period than if we had used pentobarbital sodium and because this is the least invasive and preferred euthanasia method presently available. Nevertheless, all animals were subjected to the same degree of anesthesia, and changes that we detected in respiration were nearly identical to those previously reported by Fannin et al. (16), although lower than some of our laboratory's previous studies (15, 19, 36).

H_2O_2 production. To determine whether long-term voluntary wheel running reduced mitochondrial oxidant production, we measured H_2O_2 production from freshly isolated SSM and IFM. Voluntary wheel running resulted in a significant decrease in both SSM and IFM H_2O_2 production compared with sedentary rats (Fig. 2). Furthermore, compared with SSM, H_2O_2 production from IFM was significantly decreased in both sedentary and wheel-running rats.

Antioxidant enzyme activity and glutathione levels. Voluntary exercise resulted in a significant reduction in MnSOD activity in SSM and IFM (Fig. 3*A*). In both the sedentary and running groups, MnSOD activity was higher in IFM compared with SSM. Compared with sedentary rats, there was no effect of wheel running on GPX activity in SSM or IFM (Fig. 3*B*). However, GPX activity was higher in IFM compared with SSM in both groups (sedentary and runners) of rats. No differences in CAT activity were detected in SSM or IFM due to running activity (Fig. 3*C*). As observed with GPX activity, IFM from both groups had significantly higher CAT activity than SSM. Mitochondrial levels (SSM and IFM) of GSH were also not affected by voluntary wheel running (Fig. 4*A*), but IFM from sedentary rats and runners contained less GSH than SSM. GR activity in SSM and IFM was not affected by wheel running (Fig. 4*B*), although IFM from both groups of rats had higher GR activity compared with SSM.

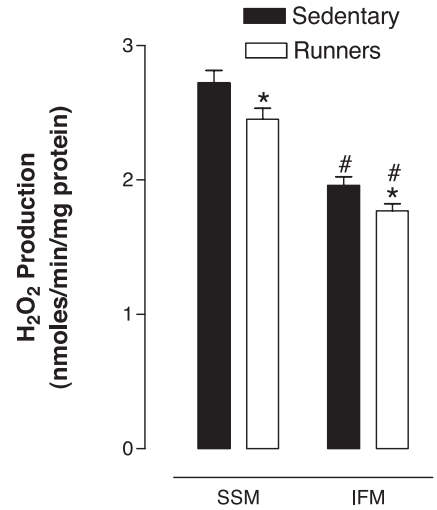

Fig. 2. Hydrogen peroxide (H_2O_2) production in subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) from sedentary and wheel-running rats. Values are means ± SE. *Significant ($P < 0.05$) difference between identical mitochondrial preparations from sedentary and wheel-running rats. #Significant ($P < 0.05$) difference between SSM and IFM within the same group of animals. For both sedentary rats and runners, $n = 12$ rats for SSM and IFM.

In contrast to what was observed in the mitochondria, there were no changes in cytosolic SOD, GPX, or CAT activities (Table 2). In addition, we observed no differences in GSH, GSSG, the GSH-to-GSSG ratio, or GR activity in the cytosol of sedentary rats and runners (Table 2).

Oxidative damage. Using Western blotting, we measured the amount of HNE-modified proteins as a specific marker of lipid peroxidation. No significant differences in the amount of HNE-modified proteins were detected in cytosol (Fig. 5*A*), SSM (Fig. 5*B*), or IFM (Fig. 5*C*) from sedentary rats and runners. Compared with sedentary rats, there was a small, but significant, increase in protein carbonyls in SSM from runners, whereas no differences between the two groups were detected in IFM (Table 3). Protein carbonyls were higher in IFM compared with SSM in both groups of rats. In agreement with

Table 1. *Effect of voluntary wheel running on mitochondrial protein yield and respiratory parameters*

	SED		WR	
	SSM	IFM	SSM	IFM
Protein yield, mg/g wet wt	16.32±0.38	9.69±0.27*	16.93±0.59	9.65±0.34*
State 4, nmol O_2 consumed·min^{-1}·mg protein^{-1}	10.94±0.71	10.99±0.99	11.39±1.02	11.73±1.20
State 3, nmol O_2 consumed·min^{-1}·mg protein^{-1}	33.33±2.09	41.60±3.53	30.24±2.89	35.06±2.88
RCR	3.02±0.22	3.36±0.40	2.85±0.25	3.25±0.35

Results are expressed as means ± SE. For both sedentary (SED) and wheel-running (WR) rats, $n = 12$ for subsarcolemmal (SSM), interfibrillar (IFM) mitochondria. Respiratory control ratio (RCR) is calculated as the ratio between state 3 and state 4 respiration. *Significant ($P < 0.05$) difference between SSM and IFM within the same group of animals.

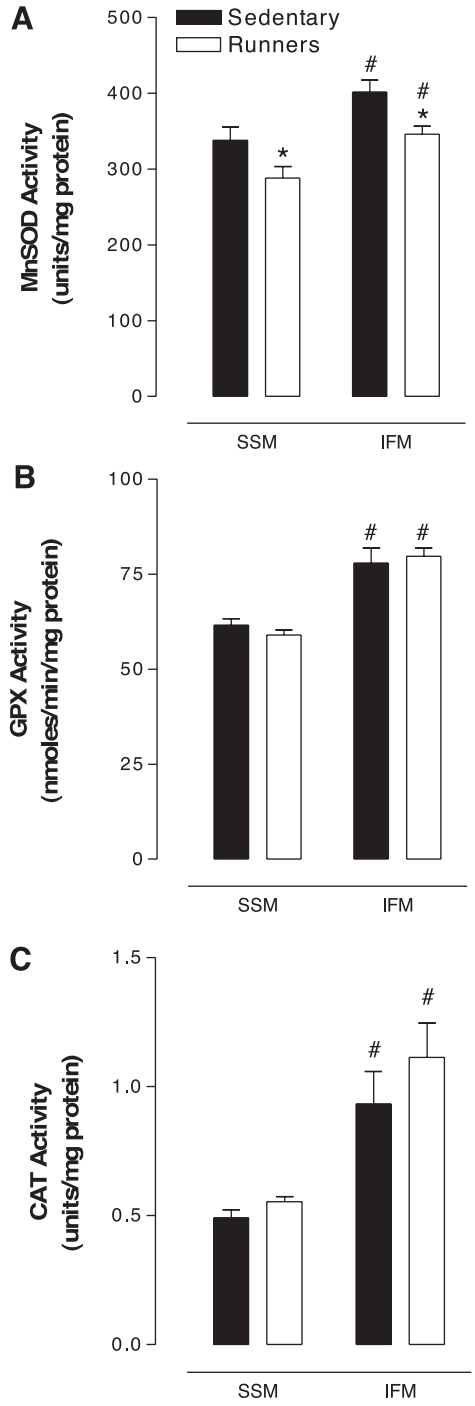

Fig. 3. MnSOD (*A*), glutathione peroxidase (GPX; *B*), and catalase (CAT; *C*) activities in SSM and IFM from the sedentary and runners groups. Values are means ± SE. *Significant ($P < 0.05$) difference between identical mitochondrial preparations from sedentary and wheel-running rats. #Significant ($P < 0.05$) difference between SSM and IFM within the same group of animals. For both groups of rats, $n = 11$ or 12 for SSM and $n = 9$–12 rats for IFM.

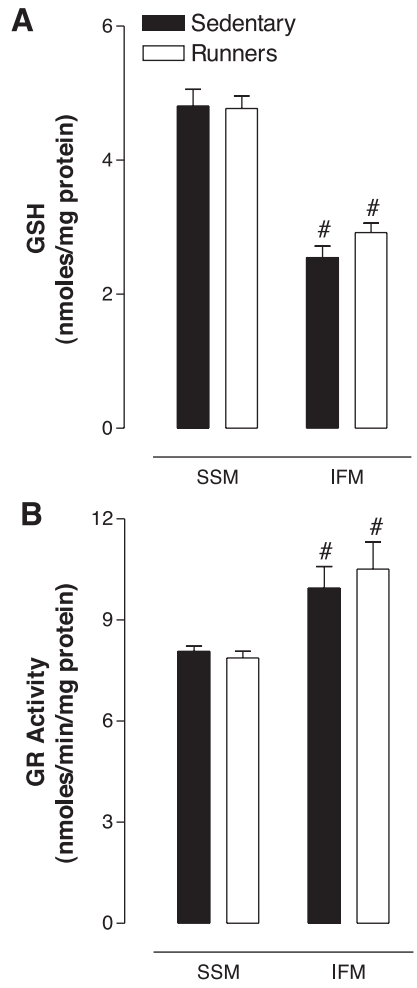

Fig. 4. Reduced glutathione (GSH) content (*A*) and glutathione reductase (GR) activity (*B*) in SSM and IFM from sedentary and wheel-running rats. Values are means ± SE. #Significant ($P < 0.05$) difference between SSM and IFM within the same group of animals. For both groups of rats, $n = 10$–12 rats for SSM and IFM.

the HNE data, no significant differences in TBARS in SSM and IFM from sedentary rats and runners were observed (Table 3). However, TBARS were significantly increased in IFM from both groups compared with SSM. Hence, lifelong exercise had no significant effect on oxidative stress levels (except for a small increase in protein carbonyls in SSM), whereas IFM from both groups of rats exhibited much greater levels of oxidative stress compared with SSM (Fig. 6).

DISCUSSION

Most lifelong, voluntary wheel-running studies have been performed for the sole purpose of determining its effects on

Table 2. *Effect of long-term voluntary wheel running on cytosolic SOD, GPX, GR, catalase, GSH, and GSSG*

	SED	WR
SOD, U/mg protein	367.9±13.0	350.9±9.4
GPX, nmol·min^{-1}·mg protein^{-1}	88.4±2.5	89.2±2.4
Catalase U/mg protein	0.80±0.07	0.83±0.05
GR, nmol·min^{-1}·mg protein^{-1}	7.85±0.31	7.6±0.22
GSH, nmol/mg protein	17.89±0.49	18.36±0.49
GSSG, nmol/mg protein	0.298±0.04	0.303±0.01
GSH:GSSG	68.61±6.82	61.43±2.11

Results are expressed as means ± SE. For SED, $n = 12$; for WR, $n = 11$. GSH:GSSG is calculated as the ratio of reduced to oxidized glutathione. GPX, glutathione peroxidase; GR, glutathione reductase; GSH and GSSG, reduced and oxidized glutathione, respectively.

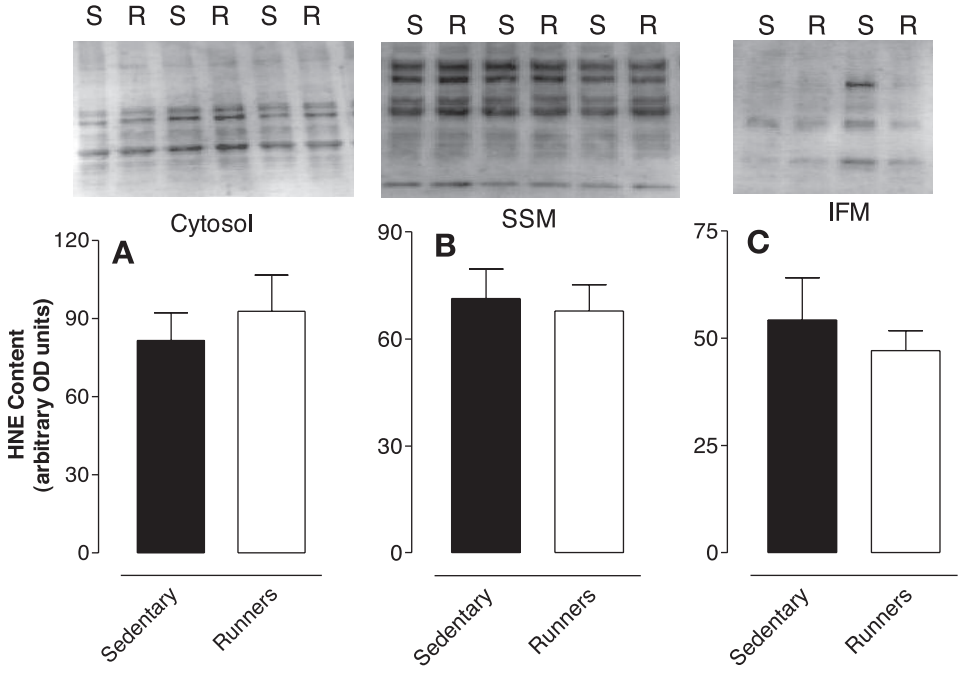

Fig. 5. Western blot analysis of 4-hydroxy-2-nonenal-modified proteins in cytosol (*A*), SSM (*B*), and IFM (*C*) from sedentary and wheel-running rats. *Top*: representative blots of HNE-modified proteins from sedentary (S) and wheel-running (R) rats. *Bottom*: graphical representations of optical densities (OD) of all the HNE-modified bands within a given lane, calculated using Kodak 1D image analysis software. Values are means ± SE. For each group, $n = 5$ rats.

lifespan. Studies performed thus far, in which food intake was carefully controlled, have only been able to show an increase in mean lifespan, with no change in maximum lifespan (23, 24). Furthermore, only a handful of studies using long-term wheel running (>18 mo) have measured any parameters of oxidative stress (29, 30, 33); to our knowledge, none has determined the effects on oxidant production. We determined whether long-term voluntary wheel running could reduce oxidant production, enhance antioxidant defenses, and reduce oxidative damage in two populations of heart mitochondria with distinct metabolic demands.

Although wheel running reduced mitochondrial H_2O_2 production, it had no effect on mitochondrial protein yield, rates of oxygen consumption (states 4 and 3), or RCR. This is not entirely surprising given that several investigators have shown that, unlike skeletal muscle, oxidative capacity of cardiac muscle is not increased in response to treadmill training (35, 49, 50), an exercise protocol that is typically much more intense than voluntary wheel running. Indeed, Bizeau et al. (7) demonstrated that 6 wk of treadmill training significantly increased state 3 respiration in skeletal muscle SSM and IFM, whereas Servais et al. (55) found that 5 mo of voluntary wheel running did not alter state 3 respiration in SSM or IFM isolated from quadriceps muscle. Because the heart is already highly oxidative, consuming more oxygen per gram of muscle at rest than skeletal muscle does during heavy exercise (2), it is not expected to be as responsive to exercise-induced increases in oxidative capacity as skeletal muscle, despite increasing overall DEE by over 70% compared with pair-fed sedentary controls.

A reduction in mitochondrial H_2O_2 production after exercise training has been reported in skeletal muscle (64), and 5 wk of voluntary running reduced levels of 2,3-dihydroxybenzoic acid (an indicator of hydroxyl radical) in heart (44). However, to our knowledge, we are the first to report a significant reduction in H_2O_2 production in SSM and IFM after 21 mo of voluntary wheel running. Because mitochondrial H_2O_2 arises as a result of the dismutation of two $O_2^-\cdot$ radicals, it can be speculated that voluntary exercise reduced the amount of $O_2^-\cdot$ being produced during electron transport. In further support of this notion, there was a significant decrease in MnSOD activity in SSM and IFM from wheel runners. Our group (28) have previously shown that MnSOD activity and H_2O_2 production are reduced in SSM from hearts of young animals after 8 wk of calorie restriction, the gold-standard life-prolonging intervention in a wide range of organisms. Lifelong calorie restriction attenuates age-related increases in mitochondrial $O_2^-\cdot$ and H_2O_2 production (3, 57), and our results support the idea that voluntary wheel running, an intervention which increases mean lifespan in rodents, may have some effects in common to calorie restriction but obviously not all, as calorie restriction extends both mean and maximum lifespan unlike voluntary exercise.

Table 3. *Protein and lipid peroxidation determined by the levels of protein carbonyls and TBARS in SSM and IFM from SED and WR rats*

	SED		WR	
	SSM	IFM	SSM	IFM
Carbonyls, nmol/mg protein	0.275±0.012	0.812±0.053†	0.320±0.007*	0.768±0.049†
TBARS, μmol/mg protein	0.575±0.031	0.939±0.038†	0.608±0.015	0.953±0.045†

Values are means ± SE. For all groups, $n = 6$. TBARS, thiobarbituric acid reactive substances. *Significant ($P < 0.05$) difference between identical mitochondrial preparations from SED and WR; †Significant ($P < 0.05$) difference between SSM and IFM within the same group of animals.

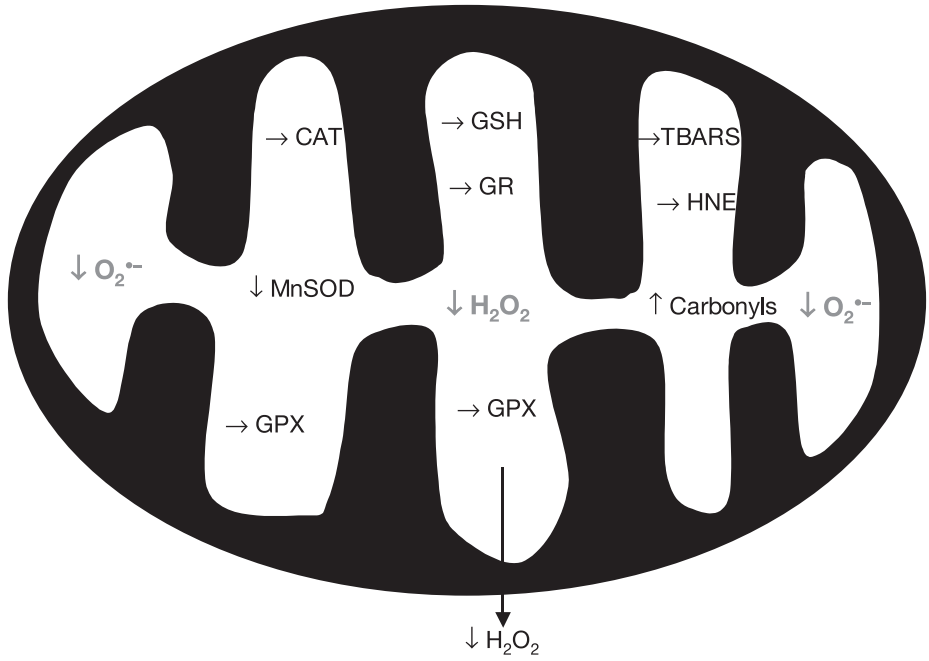

Fig. 6. Summary of changes in SSM and IFM isolated from lifelong wheel runners compared with sedentary controls. Items shown in black text are parameters that we measured, whereas items in gray text are probably occurring or measured by others (62). SSM and IFM from wheel runners likely produced less $O_2^-\cdot$, because there was a significant reduction in MnSOD activity. The decrease in the activity of this enzyme should lead to decreased production of H_2O_2 inside the mitochondria. In SSM and IFM, there were no differences between groups in mitochondrial GPX and CAT activities and, therefore, H_2O_2 released from the mitochondria was lower in wheel-running compared with sedentary animals. There were no significant changes in the levels of mitochondrial GR and GSH. Moreover, no changes were found in the levels of HNE and TBARS in SSM or IFM, but carbonyls increased in SSM from runners.

However, calorie restriction may be difficult to achieve in human societies, but voluntary exercise is easier to achieve throughout one's lifespan and has beneficial effects on cardiac health.

Wheel running did not alter the activities of any other mitochondrial or cytosolic antioxidant enzymes measured (GPX, CAT, and GR) and mitochondrial GSH levels were also not different between controls and runners. It is difficult to directly compare our results with other studies that have used exercise training (treadmill or swimming), since the duration and intensities of those types of exercise are typically much greater than voluntary wheel running and are generally performed over much shorter time periods. Kim et al. (30) measured various cytosolic antioxidant enzyme activities in hearts from 20-mo-old Fischer 344 rats after 18.5 mo of voluntary wheel running. Similar to our results, they found no effect of wheel running on cytosolic SOD or GPX, but they did find a significant increase in cytosolic CAT activity. Another study examining the effects of short-term voluntary wheel running (1 and 7 days) found results remarkably similar to ours in that total SOD activity was decreased in the hearts of 1- and 7-day runners, whereas GPX and CAT activity remained unchanged (54). Although the reduction in MnSOD activity in wheel running animals may be in response to reduced mitochondrial $O_2^-\cdot$, additional studies are required to further substantiate this. Because $O_2^-\cdot$ production cannot be measured in intact mitochondria and must be measured in submitochondrial particles, which may not represent respiratory function of intact mitochondria, H_2O_2 production is often used as a measure of mitochondrial oxidant production and is believed to be an accurate reflection of mitochondrial $O_2^-\cdot$ production (3–5, 34). However, without directly measuring superoxide production, definitive conclusions cannot be made regarding the quantitative levels of $O_2^-\cdot$ measured under state 4 basal conditions within isolated mitochondria.

Voluntary wheel running did not reduce the amount of HNE-modified proteins in cytosol, SSM, or IFM, nor did it reduce the amount of TBARS in SSM or IFM. Navarro et al. (41) examined oxidative damage in hearts of mice that performed moderate treadmill exercise from 28 to 78 wk of age. At 52 wk, protein carbonyls and TBARS were significantly reduced in hearts of exercised mice compared with age-matched sedentary controls. However, at 78 wk, no differences in carbonyls or TBARS were observed between the exercised and sedentary mice. Because our rats were 104 wk old at the time of death, this may be one reason why we found no differences in lipid peroxidation between sedentary and wheel-running rats. Together, these studies strongly suggest that moderate exercise reduces oxidative damage in adult rodents, although, with advancing age, these benefits are no longer observed. Hence, the lack of effect on oxidative stress markers in our study may be partly due to the age of the animals at death.

We observed a small (12%) but significant increase in protein carbonyls in SSM from wheel-running rats compared with sedentary rats. It is possible that this increase is related to the significant reduction in MnSOD activity in SSM observed in runners. Our group (28) previously reported that, despite a reduction in H_2O_2 production and MnSOD activity, protein carbonyls in SSM from calorie-restricted rats were increased compared with ad libitum fed rats. Bota et al. (8) found that young (3–6 mo) *Sod2*$^{+/-}$ mice, which exhibit a 50% decrease in MnSOD activity, display an 80% increase in skeletal muscle protein carbonyls compared with young *Sod2*$^{+/+}$ mice. Old (27 mo) *Sod2*$^{+/-}$ mice had an even greater increase in carbonyls (250%) compared with young *Sod2*$^{+/+}$ mice. Although MnSOD activity was also reduced in IFM from runners, no significant differences in carbonyls in IFM were observed between sedentary and wheel-running rats. This may be partially explained by the fact that IFM had higher antioxidant enzyme activity compared with SSM.

The aim that we set out to test was that long-term voluntary wheel running would reduce mitochondrial oxidant production and oxidative damage. The extent to which oxidized macromolecules accumulate is dependent on several factors, includ-

ing the amount and type of oxidant produced, the ability of the antioxidant defense system to prevent oxidant-induced damage, and the cell's capacity to repair or remove oxidized DNA, lipids, and proteins. Unfortunately, it was not feasible to measure every aspect of the extensive antioxidant defense system, so we chose to measure the major antioxidant enzymes (SOD, CAT, and GPX) and one of the important low-molecular weight antioxidants (GSH) and the enzyme primarily responsible for reducing it (GR). Future studies need to investigate other molecules [i.e., ascorbate, NAD(P)H] and enzymes (i.e., thioredoxin reductase, glutaredoxin reductase), which are all important in maintaining the redox environment of the mitochondria. It is impossible to isolate enough mitochondria to measure all of the systems involved in maintaining redox balance. Furthermore, there is no widely available "gold-standard" method for measuring oxidative stress. Therefore, we chose to use several markers of oxidative stress (carbonyls, HNE, and TBARS) to gain a better understanding of the effects of wheel running. The no-effect findings do not necessarily imply that the methods that we used were not sensitive enough, and we have discussed other possible reasons, such as the age of the animals, turnover of biomarkers, alternative structural targets, as to why we saw no effect. Future studies need to address specific questions related to alternative targets (i.e., DNA) or biomolecules (enzymes). We feel that our findings are very novel and timely, seeing as there are no reports in the literature regarding the effects of exercise by long-term wheel running on mitochondrial function, oxidant production, and antioxidant defenses in the heart.

It is possible that the very mild food restriction (8% below ad libitum intake) imposed on both our wheel-running and sedentary rats may have independently exerted positive effects in the sedentary group that masked additional beneficial effects of the wheel running. Holloszy (24) reported that the beneficial effects of calorie restriction (70% of ad libitum intake) and wheel running were not additive or synergistic in terms of lifespan; calorie-restricted, wheel-running rats had the same maximal lifespan as calorie-restricted, sedentary rats. Therefore, it is likely that the modest food restriction used in our study may have hindered our ability to detect additional beneficial effects that occurred as a result of wheel running. However, because wheel running has been reported to increase mean lifespan in rodents (22, 24–26), it is clear that additional studies are needed to further characterize the mechanisms underlying this observation. From our findings, we cannot rule out the possibility that reduced mitochondrial oxidant production is one contributing factor for the increase in mean lifespan observed in wheel-running rodents and may be an important mechanistic factor in the life-enhancing effects of voluntary exercise.

In summary, the reduction in chronic oxidant production may have had a beneficial effect earlier in the wheel runner's lifespan and may explain the increase in mean lifespan observed in wheel-running rodents. However, at 24-mo of age, no major differences in oxidative stress were detected between the two groups, a finding that might help to explain why lifelong voluntary exercise extends mean, but not maximum, lifespan.

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