ANTIOXIDANTS & REDOX SIGNALING
Volume 8, Numbers 3 & 4, 2006

Forum Original Research Communication

Moderate Caloric Restriction Increases Diaphragmatic Antioxidant Enzyme mRNA, but Not When Combined with Lifelong Exercise

KEITH C. DERUISSEAU,[1,4] ANDREAS N. KAVAZIS,[1,4] SHARON JUDGE,[2] ZSOLT MURLASITS,[1] MELISSA A. DEERING,[1] JOHN C. QUINDRY,[1] YOUNGIL LEE,[1] DARIN J. FALK,[1] CHRISTIAAN LEEUWENBURGH,[3] and SCOTT K. POWERS[1]

ABSTRACT

Diaphragmatic antioxidant enzymes are upregulated following acute and long-term treadmill exercise, but the effect of lifelong voluntary exercise (E) on diaphragmatic antioxidants is unknown. Therefore, 10-week old Fisher 344 rats were assigned to either: (a) sedentary *ad libitum* (AL) fed (24AL; *n* = 6); (b) E + 8% caloric restriction (24ECR; *n* = 9); or (c) sedentary + 8% caloric restriction (24CR; *n* = 9) groups. Diaphragms were harvested from animals at 24 months of age. Heme oxygenase-1 (HO-1) mRNA in addition to catalase (CAT), glutathione peroxidase (GPX), copper-zinc superoxide dismutase (Cu-ZnSOD) and manganese superoxide dismutase (MnSOD) mRNA and protein levels were measured. Reduced glutathione (GSH) and citrate synthase (CS) activity were measured to assess antioxidant status and oxidative capacity, respectively. The 24CR group demonstrated increased GPX, HO-1, MnSOD, and CAT mRNA compared to 24AL and 24ECR. Interestingly, the increased mRNA in 24CR animals did not result in elevated protein levels. No group differences in Cu-ZnSOD mRNA, CS activity, or GSH were observed, although GSH was 30% greater in 24CR animals (*p* = 0.085). In summary, although CR elevated the mRNA of key antioxidant enzymes in the diaphragm, lifelong CR alone or in combination with voluntary exercise did not alter diaphragm CS activity, antioxidant protein quantity, or GSH levels. *Antioxid. Redox Signal.* 8, 539–547.

INTRODUCTION

THE ANTIOXIDANT SYSTEM of mammalian cells involves complex interactions between enzymatic and nonenzymatic compounds. Antioxidants play a central role in the regulation of the intracellular redox balance and provide a vital defense against the potentially damaging effects of reactive oxygen species (ROS). Important antioxidant enzymes include copper-zinc and manganese superoxide dismutase (Cu-ZnSOD and MnSOD, respectively), catalase (CAT), and glutathione peroxidase (GPX). Heme oxygenase-1 (HO-1), although not a direct scavenger of ROS, degrades heme proteins and thus facilitates the removal of reactive iron and leads to the formation of the bile pigment, bilirubin (4). Additionally, glutathione is a major nonenzymatic antioxidant and is the most prevalent intracellular nonprotein thiol (20).

Acute exercise (i.e., muscle contraction) results in elevated intracellular ROS production (26) and release (27, 34) by

[1]Department of Applied Physiology and Kinesiology, University of Florida, Gainesville, Florida.
[2]Myocardial Biology Unit, Boston University School of Medicine, Boston, Massachusetts.
[3]Department of Aging and Geriatric Research, College of Medicine, University of Florida Institute on Aging, Biochemistry of Aging Laboratory, Gainesville, Florida.
[4]Authors contributed equally to this work.

skeletal muscle. Importantly, the antioxidant status of skeletal muscle can be upregulated by both short- and long-term exercise. Moreover, increased skeletal muscle ROS formation is generally thought to be a key mediator of exercise-induced antioxidant upregulation (12). Therefore, the increased antioxidant capacity of skeletal muscle observed following exercise is likely a protective adaptation to attenuate the potentially damaging effects of exercise-induced ROS production.

The mammalian diaphragm is a unique and chronically active skeletal muscle that exhibits an increased antioxidant status following both short- (days) and long- (weeks-to-months) term treadmill exercise (24, 36, 37) and swim training (23). Most previous studies that have examined the impact of exercise on the skeletal muscle antioxidant status have utilized forced exercise (e.g., treadmill or swim training) for periods ranging from days to a year (3, 8, 9, 14, 21, 23, 24, 36, 37). Although several studies have investigated the impact of short- and long-term voluntary exercise on the antioxidant response in locomotor muscle (16, 28, 29), the effect of lifelong voluntary exercise on the antioxidant response of the diaphragm is currently unknown. Additionally, only limited data characterize the mRNA response of key antioxidant enzymes in the diaphragm following exercise training (11, 22).

Therefore, the purpose of this experiment was to examine the effects of lifelong wheel running exercise on key antioxidant enzymes in the diaphragm. In these experiments we utilized male Fisher 344 rats, a well-accepted model used to examine the skeletal muscle antioxidant response to both exercise and aging. We imposed a moderate food restriction (8% below *ad libitum* fed) on animals in the exercise group since this has been shown to prevent the decline in spontaneous wheel running activity observed in rats fed *ad libitum* (10). Additionally, we imposed the same degree of food restriction (8%) on a group of sedentary animals to serve as dietary intake controls. We hypothesized that lifelong voluntary exercise would increase mRNA expression of key antioxidants and elevate the antioxidant status of the diaphragm. Specifically, we postulated that lifelong wheel running exercise would increase the mRNA levels of Cu-ZnSOD, MnSOD, CAT, GPX, and HO-1. Additionally, we postulated that changes in mRNA levels would result in increased protein levels of Cu-ZnSOD, MnSOD, CAT, and GPX. Furthermore, we postulated that lifelong wheel running exercise would elevate the level of reduced glutathione (GSH) in the diaphragm, which would be indicative of an enhanced nonenzymatic antioxidant status.

Our results indicate that lifelong voluntary exercise combined with moderate caloric restriction (CR) did not alter diaphragmatic antioxidant mRNA expression or GSH levels. In contrast, the diaphragms of sedentary rats consuming lifelong calorie-restricted diets increased mRNA expression of several antioxidants and demonstrated a trend towards elevated levels of GSH compared to sedentary *ad libitum* fed animals. Nonetheless, protein levels of antioxidant enzymes did not mirror changes in mRNA expression. Our findings suggest that an enhanced antioxidant status and upregulated gene expression of key antioxidant enzymes develops in the diaphragm following lifelong CR in sedentary animals, but not when CR is combined with lifelong voluntary exercise.

MATERIALS AND METHODS

Experimental animals and design

Male Fisher 344 rats were obtained from Harlan (Indianapolis, IN) and were assigned to one of three groups: (a) sedentary *ad libitum* fed (24AL; $n = 6$); (b) sedentary with 8% caloric restriction (24CR; $n = 9$); and (c) lifelong wheel running exercise with 8% caloric restriction (24ECR; $n = 9$). Animals in the 24AL, 24CR, and 24ECR groups arrived at 10 weeks of age and were allowed to age in our facilities until they were 24 months of age.

Since rats that are fed *ad libitum* tend to abruptly decrease their running activity at approximately 6 months of age (10, 19), we applied a moderate food restriction (8% below *ad libitum* fed) to 24ECR animals as this has been shown to prevent the decline in spontaneous wheel running activity (10). Animals in the 24CR group served as sedentary calorie-restricted controls. Therefore, food intake for the 24CR and 24ECR groups was restricted by 8% below the *ad libitum* food intake of 24AL group. Throughout the duration of the study, food intake of the 24CR and 24ECR groups was adjusted accordingly each week (based on *ad libitum* food intake of the animals in the 24AL group from the previous week). Animals were fed rat chow (Harlan Teklad Rodent Diet #8604) and all animals had unrestricted access to water.

All animals were singly housed in a temperature (20 ± 2.5°C) and light-controlled (12:12-h light–dark cycle) environment. 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 meters 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 daily number of wheel revolutions. Animals were euthanized with isoflurane (administered via inhalation using a precision vaporizer at 5%), and diaphragms were rapidly removed. Segments from the costal region were rapidly frozen in liquid nitrogen and stored at −80°C for subsequent analysis. All experimental procedures were approved by the University of Florida's Institute on Animal Care and Use Committee.

Isolation of total RNA

A portion of the costal diaphragm (~60 mg) was homogenized in 1.5 ml of Trizol reagent (Invitrogen, Carlsbad, CA) and processed according to the manufacturer's instructions. Samples were homogenized with a Polytron blade homogenizer on ice at medium speed and centrifuged at 12,000 *g* for 10 min (4°C) to remove insoluble material. The supernatant was transferred and RNA was extracted with 120 μl of bromochloropropane. Samples were vortexed briefly and centrifuged at 13,000 *g* for 20 min (4°C). Following transfer of the aqueous phase, RNA was precipitated with one volume of isopropanol and washed twice with two volumes of 75% ethanol. The RNA was pelleted via centrifugation and resuspended in RNase-free water (Sigma, St. Louis, MO). Concentration and purity of the extracted RNA was measured spectrophotometrically at 260 nm and at 280 nm in 1× TE buffer

(Promega, Madison, WI). The integrity of the extracted total RNA was verified by gel electrophoresis of 1 μg RNA on a 1% agarose ethidium bromide-stained TBE gel. Total RNA was stored at −80°C.

Reverse transcription and cDNA quantification

Reverse transcription (RT) was performed using the Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Reactions were carried out using 5 μg of total RNA and 2.5 μ*M* oligo(dT)$_{20}$ primers. First strand cDNA was subsequently treated with 2 units of RNase H. Following the addition of 2 μl of GlycoBlue coprecipitant (Ambion) to the RT product (21 μl), first strand cDNA was purified of RNA and unincorporated nucleotides by treatment with an RNase cocktail (Ambion), brought to a volume of 100 μl with nuclease-free water (Sigma), and applied to a NucAway spin column (Ambion). Samples were then mixed with phenol:chloroform:isoamyl alcohol (pH = 7.9) and the aqueous phase recovered using a 1.5 ml heavy phase lock gel (Eppendorf, Hamburg, Germany). The cDNA was precipitated by adding one volume of 5.0 *M* ammonium acetate and two volumes of 100% ethanol and stored at −20°C overnight. Following centrifugation at 14,000 *g* for 20 min (4°C) the cDNA was washed with two volumes of 75% ethanol, centrifuged at 14,000 *g* for 10 min (4°C) and resuspended in 50 μl of 1× TE buffer. The cDNA was subsequently quantified using the Oligreen single strand DNA Quantitation Reagent and Kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR).

Quantitative real-time PCR

Quantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detection System (ABI, Foster City, CA). The probes for all genes consisted of Taqman 5′ labeled FAM reporters and 3′ nonfluorescent quenchers. Primers and probes for Cu-ZnSOD (GenBank mRNA; M21060, Y00404, BC082800, BC058148, X05634), MnSOD (GenBank mRNA; Y00497, BC070913), CAT (GenBank mRNA; BC081853, M11670), and GPX (GenBank mRNA; S41066, BC058438, M21210, X12367, X07365) were obtained from Applied Biosystems (Assays-on-Demand; ABI, Foster City, CA). The sequences used by the manufacturer in the design of primers and probes from this service are proprietary and are therefore not reported. However, the context sequences (i.e., the nucleotide sequence surrounding the probe) are available and consist of the following: Cu-ZnSOD, 5′-AGCAGAAGGCAAGCGGTGAACCAGT-3′; MnSOD, 5′-CGGGCGGCGTGCAGCGCGGGCAGAA-3′; CAT, 5′-CAGCGGGCCCCTCAGAAACCCGATG-3′; and GPX, 5′-AGTTCGGACATCAGGAGAATGGCAA-3′. The heme oxygenase-1 (HO-1) and hypoxanthine guanine phosphoribosyl transferase (HPRT) primers and probes were obtained from Applied Biosystems (Assays-by-Design). Primer and probe sequences for HO-1 are: Forward, 5′-GGTACGTGACGTGTAGGTTGAC-3′; Reverse, 5′-GCACCAACCTCCCCATTAAACTATT-3′; Probe, 5′-CTTAAGCCCTGGGTATACC-3′. Primer and probe sequences for HPRT are: Forward, 5′-GTTGGATACAGGCCAGACTTTGT-3′; Reverse, 5′-AGTCAAGGGCATATCCAACAACAA-3′; Probe, 5′-ACTTGTCTGGAATTTCA-3′. Each 25 μl PCR reaction, performed in duplicate, contained 3.0 ng of cDNA template. Gene expression was calculated using the relative standard curve method as described in the ABI, User Bulletin #2 (ABI, Foster City, CA). HPRT was selected as the appropriate normalizer since the expression of this gene in the diaphragm was not significantly altered by the experimental treatments ($p = 0.46$).

Western blot analysis

A section (50–75 mg) of the costal diaphragm was homogenized and assayed to quantitatively determine the protein levels of Cu-ZnSOD, MnSOD, CAT, and GPX. Samples were homogenized 1:10 (w/v) (5 m*M* Tris HCl, pH = 7.5, 5 m*M* EDTA) and centrifuged at 1500 *g* for 10 min (4°C). The supernatant (cytosolic fraction) was centrifuged at 10,000 *g* for 10 min (4°C) followed by an additional spin of the supernatant at 100,000 *g* for 1 h (4°C). Protein content of the cytosolic fraction was assessed by the method of Bradford (Sigma). Proteins (36 μg) from the cytosolic fraction were then separated by polyacrylamide gel electrophoresis via 10% gradient polyacrylamide gels containing 0.1% sodium dodecyl sulfate. After electrophoresis, the proteins were transferred to nitrocellulose membranes (200 mA for 1.5 h). Membranes were stained with Ponceau S and visually inspected for equal protein loading and transfer. The membranes were then washed and blocked in PBS-Tween buffer containing 5.0% skim milk and 0.05% Tween for 2 h and subsequently incubated with a primary antibody directed against Cu-ZnSOD (SOD-101; Stressgen, Victoria, Canada), MnSOD (SOD-111; Stressgen), CAT (ab16731; Abcam; Cambridge, MA) or GPX (ab16798; Abcam). Primary antibodies were diluted 1:1000 for Cu-ZnSOD and 1:2000 for MnSOD, CAT, and GPX in blocking buffer and applied to the membranes overnight at 4°C. This step was followed by incubation with a horseradish peroxidase-antibody conjugate (1:2,000) directed against the primary antibody for 1 h. The membranes were then treated with chemiluminescent reagents (luminol and enhancer) and exposed to light-sensitive film. Images of these films were captured and analyzed using the 440CF Kodak Imaging System (Kodak, New Haven, CT).

Citrate synthase (CS) activity

Citrate synthase activity [CS Enzyme Commission no. (EC) 2.3.3.1] was utilized as a marker of oxidative capacity and was measured according to the method described by Srere (32).

Reduced glutathione levels

Reduced glutathione (GSH) levels were measured as an indicator of antioxidant status using a commercially available kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Statistical methods

Data are presented as means ± standard error of the mean. Statistical significance between groups for each dependent

variable was determined by a one-way analysis of variance with Scheffé post-hoc analysis. Significance was established at $p < 0.05$.

RESULTS

Animal body weights and wheel running activity

The 24ECR animals (338.0 ± 6.8 g) weighed less ($p < 0.05$) than 24AL (407.0 ± 11.8 g) and 24CR animals (377.5 ± 6.6 g). The difference in body weight between the 24AL and 24CR groups did not reach statistical significance ($p = 0.07$). Running activity of the 24ECR animals is illustrated in Figure 1. Running activity for this group averaged 677 m/day during the first week and peaked at 3300 m/day at 6 months of age. Running activity was approximately 1600 m/day at 1 year and declined slightly to approximately 1300 m/day for the remainder of the study. Running data are not reported for the final month since animals were sacrificed between days 7 and 12.

Diaphragmatic mRNA levels of antioxidant enzymes

Table 1 contains diaphragmatic mRNA expression levels for genes assessed by quantitative real-time PCR. The diaphragms of sedentary animals fed the food-restricted diet (24CR) displayed elevated ($p < 0.05$) MnSOD, CAT, GPX, and HO-1 mRNA expression levels compared to the sedentary *ad libitum* fed (24AL) and exercise + 8% caloric restriction (24ECR) groups. No changes were detected in Cu-ZnSOD mRNA expression between any of the experimental groups.

Diaphragmatic protein levels of antioxidant enzymes

Protein levels of CAT, Cu-ZnSOD, MnSOD, and GPX were determined by Western blot (Fig. 2). No significant change in the arbitrary optical density units for any of the proteins examined was observed between the 24AL, 24ECR, and 24CR groups.

Diaphragmatic reduced glutathione (GSH) levels and citrate synthase (CS) activity

GSH levels are depicted in Figure 3A. The animals in the sedentary calorie-restricted group (24CR) demonstrated a 32.3% increase in GSH levels compared to 24AL animals and a 26.5% increase in GSH levels compared to 24ECR animals. One-way ANOVA indicated a significant *F*-ratio between the 24AL, 24ECR, and 24CR groups ($p = 0.042$). However, the difference between the 24CR and 24AL groups did not reach statistical significance upon post-hoc analysis ($p = 0.085$).

Citrate synthase activity is illustrated in Figure 3B. No difference ($p > 0.05$) in the diaphragm CS activity was detected between the 24AL, 24ERC, and 24CR groups.

DISCUSSION

Overview of principal findings

These experiments provide new and important information regarding the impact of lifelong voluntary exercise and moderate caloric restriction (8% below *ad libitum* intake) on the antioxidant status of the rat diaphragm. To our knowledge, no studies have previously reported the diaphragmatic antiox-

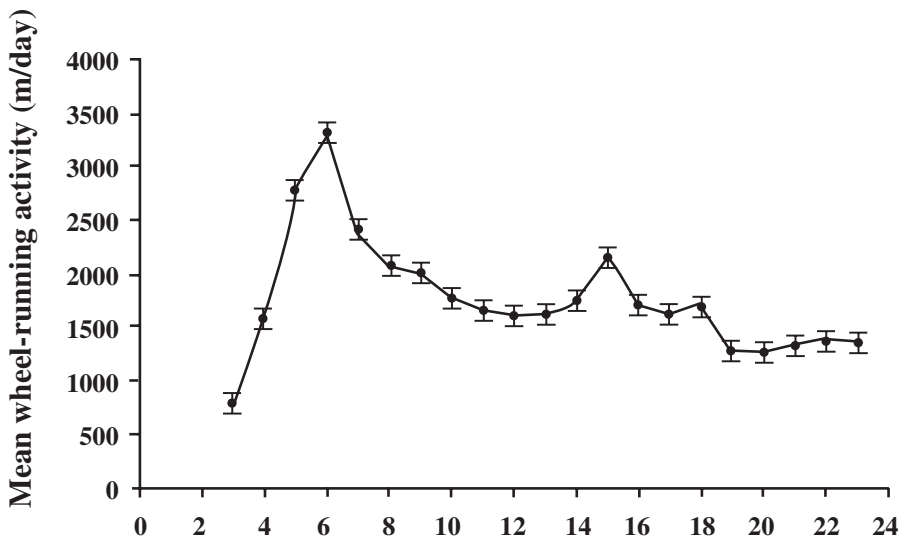

FIG. 1. Running wheel activity data for animals in the exercise + 8% caloric restriction (24ECR) group. Wheel-running activity is presented for each month and is expressed as average meters run per day.

TABLE 1. DIAPHRAGM ANTIOXIDANT mRNA ASSESSED BY QUANTITATIVE REAL-TIME PCR

mRNA	*24AL*	*24ECR*	*24CR*
Cu-ZnSOD	1.59 ± 0.10	1.24 ± 0.10	1.51 ± 0.08
MnSOD	0.25 ± 0.05	0.30 ± 0.03	0.46 ± 0.03*
CAT	0.48 ± 0.19	0.62 ± 0.09	1.35 ± 0.15*
GPX	0.57 ± 0.11	0.66 ± 0.06	0.92 ± 0.05*
HO-1	81.07 ± 41.03	120.48 ± 19.09	276.58 ± 44.62*
HPRT	3.47 ± 0.21	3.69 ± 0.17	3.76 ± 0.22

Values are means ± standard error of the mean.
*Significantly different from 24AL and 24ECR groups ($p < 0.05$).
See Abbreviations list for definitions.

idant enzyme mRNA response of animals to lifelong exercise training. Contrary to our hypothesis, lifelong voluntary exercise did not increase diaphragmatic mRNA expression of key antioxidant enzymes or elevate GSH levels of the costal diaphragm. However, these experiments did reveal the novel finding that antioxidant enzyme mRNA of the diaphragm is upregulated by a modest reduction in lifelong caloric intake. Moreover, when combined with lifelong voluntary wheel run-

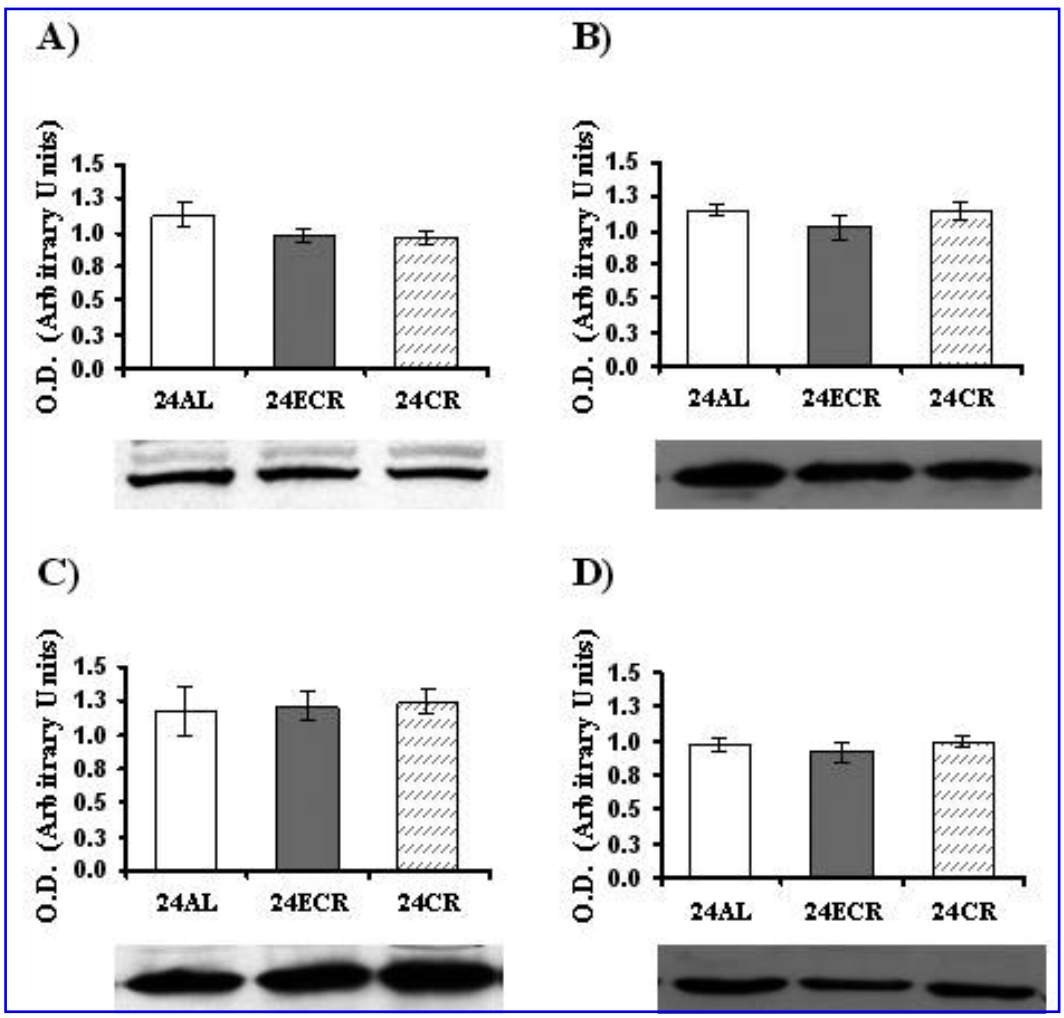

FIG. 2. Densiometric analysis and representative Western blots of antioxidant protein levels in the diaphragm. (**A**) Catalase (CAT); (**B**) Copper-zinc superoxide dismutase (Cu-ZnSOD); (**C**) Manganese superoxide dismutase (MnSOD); (**D**) Glutathione peroxidase (GPX). 24AL, 24-month-old sedentary *ad libitum* fed; 24ECR, 24-month-old exercise + 8% caloric restriction; 24CR, 24- month-old sedentary + 8% caloric restriction.

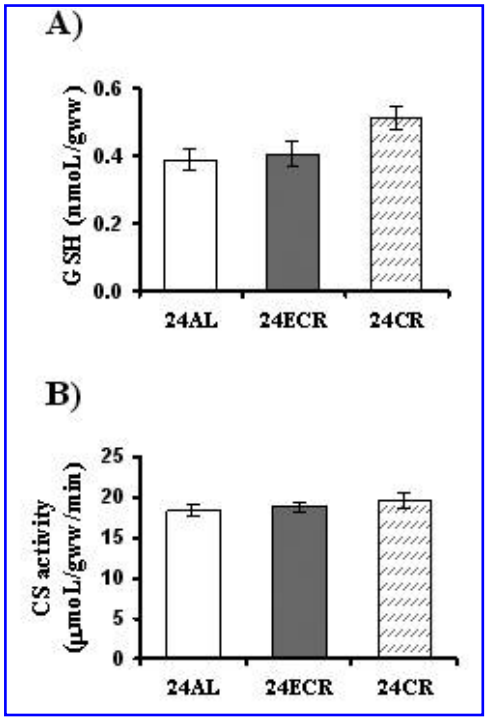

FIG. 3. Reduced glutathione (GSH) levels and citrate synthase (CS) activity in the diaphragm. (**A**) GSH levels; (**B**) CS activity. 24AL, 24-month-old sedentary *ad libitum* fed; 24ECR, 24-month-old exercise + 8% caloric restriction; 24CR, 24-month-old sedentary + 8% caloric restriction.

ning exercise, the effect of CR on the diaphragmatic antioxidant response was abated.

Lifelong exercise with moderate caloric restriction does not alter diaphragmatic antioxidant status

The costal diaphragm of young adult rats exhibits an increased antioxidant status following short- and long-term treadmill exercise (24, 36, 37) and swim training (23). However, in the current experiment no exercise-induced alterations in the expression level of antioxidant mRNA or proteins were observed. Moreover, the lack of unaltered citrate synthase activity is indicative of an unchanged diaphragmatic oxidative capacity. Therefore, the lack of response in the antioxidant status of the diaphragm following lifelong voluntary exercise may be the result of an age-related reduction in the duration and intensity of voluntary exercise during the last weeks of the experiment. Indeed, the failure to observe an exercise-induced increase in diaphragmatic citrate synthase in these old animals is indicative of an unchanged oxidative capacity, which implies that the level of exercise training at the conclusion of these experiments was below the threshold required for metabolic adaptation in the diaphragm. Nonetheless, it should also be noted that the skeletal muscle antioxidant response of young animals to exercise training has been shown to be greater than the response observed in aged animals (8, 15, 23). Therefore, this may contribute to the lack of change in antioxidant enzyme expression levels of animals in the lifelong exercise group. Furthermore, potential effects of moderate CR with and without lifelong voluntary exercise on ventilation and respiratory mechanics were not examined. The effects of severe CR (i.e., semi-starvation) for periods ranging from 7 to 10 days has been reported to exert no effect on ventilatory muscle strength and endurance (2) and depressed hypoxic ventilatory drive (1, 5). It is unknown if such effects would be observed following a more moderate degree of calorie restriction, such as that utilized in the current experiment.

Differences have been reported in the literature regarding the skeletal muscle antioxidant response to voluntary exercise. For example, short-term voluntary exercise was not associated with altered skeletal muscle CAT, GPX, or total SOD activity (28). Moreover, 5 months of voluntary exercise resulted in elevated CAT and GPX activities, but no change in the activities of MnSOD or Cu-ZnSOD was observed (29). Following lifelong voluntary exercise, increased activities of MnSOD and GPX were observed in skeletal muscle, but activities of CAT and Cu-ZnSOD were not altered (16). Clearly, the antioxidant response of skeletal muscle to voluntary exercise training is variable and has not been clearly defined.

In the present study, additional possibilities that may explain the lack of altered antioxidant expression following lifelong voluntary exercise training include alterations in the rates of mRNA translation and/or altered rates of protein turnover in this group of animals. However, as shown in previous investigations, altered activities of antioxidant enzymes following exercise training can occur in the absence of changes in the levels of antioxidant mRNA and/or protein levels in the diaphragm (11, 22) and locomotor muscle (7); which may reflect the occurrence of post-translational modifications. Also, the time of animal sacrifice following the last bout of exercise may also affect levels of mRNA expression. For example, upregulation of HO-1 mRNA (+7-fold) in the plantaris has been reported immediately following acute treadmill exercise, but was not different (+1.8-fold) 24 h post-exercise (6). In the present study, animals had free access to the running wheels until the time of sacrifice.

Caloric restriction upregulates diaphragmatic antioxidant mRNA

A surprising and novel finding of this study is the observation of elevated antioxidant enzyme mRNA in the diaphragms of sedentary rats consuming a calorie-restricted diet of only 8% below the caloric intake of sedentary, *ad libitum* fed animals. In fact, caloric restriction of 5–15% below *ad libitum* intake is often imposed on control animals to prevent the development of obesity (25). We did not expect to observe significant alterations in any of the dependent measures following this moderate reduction in caloric intake. Typically, experiments investigating the effects of food restriction on the extension of maximal lifespan and other biochemical and molecular markers implement larger reductions in caloric intake (40% below *ad libitum* fed). Furthermore, a caloric deficit of 8% below *ad libitum* intake has not been shown to prolong mean and maximum lifespan of rodents (10).

The ability of lifelong, moderate CR to enhance the diaphragmatic antioxidant status of the sedentary rats observed

in the present experiment may be associated with changes in the intracellular redox status. Importantly, reduced caloric intake to 60–70% of *ad libitum* fed animals has been shown to reduce levels of skeletal muscle oxidative stress (13, 17, 38). Moreover, the protective effects of CR against oxidant-mediated damage in skeletal muscle may be conveyed through reductions in mitochondrial oxidant production or possibly through an alteration in the antioxidant status (13, 33); although across various tissues it has been noted that there is no consistent pattern in the antioxidant enzyme response to CR (30). Although the occurrence of oxidative stress was not established in the present experiment, the increased mRNA expression of several key antioxidant enzymes in the 24CR animals, in addition to an approximate 30% elevation in GSH levels ($p = 0.085$), is suggestive of an enhanced antioxidant status in the diaphragms of calorie-restricted rats. Nonetheless, despite the increased mRNA levels of key antioxidant enzymes, the protein levels of these antioxidants were not different following lifelong caloric restriction. Even though CR (30–40% below *ad libitum* fed) is generally believed to improve the intracellular oxidant status, it may be possible that lifelong moderate CR actually increased diaphragmatic oxidant production; whereby antioxidant mRNA expression was increased but did not result in elevated protein levels due to an impaired mRNA translational capacity or altered rate of protein turnover. Clearly, additional knowledge of the diaphragmatic oxidative state in response to lifelong moderate CR would assist in the elucidation of the signaling mechanism(s) involved in the regulation of altered antioxidant mRNA expression.

To our knowledge, the present study is the first to examine the antioxidant enzyme mRNA response in the diaphragms of moderate calorie-restricted animals. Studies that have reported the effects of CR on locomotor skeletal muscle antioxidant enzyme activities and mRNA expression have imposed caloric reductions on the order of 30–40% below *ad libitum* intake levels (13, 18, 31). In the gastrocnemius of rats subjected to 9 months of CR (i.e., 40% below *ad libitum* intake), upregulated mRNA expression of Cu-ZnSOD (+2.8-fold), MnSOD (+2.1-fold), and GPX (+2.1-fold) was detected compared to *ad libitum* fed controls (31). Furthermore, 2 years of moderate CR (20% below *ad libitum* intake) elevated GPX activity in the soleus muscle compared to the *ad libitum* fed controls; however, no change was detected in the activities of CAT or SOD (35). Importantly, the results of the present experiment, in conjunction with those from locomotor muscle, are in general agreement that CR is associated with altered regulation of key antioxidant enzymes in skeletal muscle.

Heme oxygenase-1 (a.k.a. HSP32), a member of the heat shock protein family, is an inducible stress response protein that appears to exhibit antioxidant properties (4). The upregulated mRNA expression of HO-1 in the 24CR animals is a notable difference between data of the current study with those obtained from locomotor skeletal muscle. Two years of moderate CR (20% below *ad libitum*) has been shown to reduce the protein expression of HO-1 in the rat soleus compared to age-matched, *ad libitum* fed controls (35). However, in the current experiment, upregulated HO-1 mRNA expression is consistent with the notion that an upregulated antioxidant response in the diaphragm is observed in response to moderate CR imposed throughout lifespan.

SUMMARY AND CONCLUSIONS

The mammalian diaphragm has the capacity to upregulate the activities of several key enzymatic antioxidants following both short- and long-term exercise. Surprisingly, lifelong voluntary exercise did not result in significantly altered diaphragmatic antioxidant mRNA, protein expression, or GSH levels. The inability of lifelong voluntary exercise to elevate citrate synthase activity, a marker of oxidative capacity, may partly explain why lifelong voluntary exercise did not elevate the diaphragmatic antioxidant status; although an unknown interaction between moderate CR and lifelong exercise may have also occurred. Unexpectedly, increased mRNA expression of several antioxidants and a trend in elevated GSH levels were detected in diaphragms of animals consuming a caloric intake 8% below the intake level of *ad libitum* fed controls. Therefore, these findings are suggestive of an upregulated antioxidant status in the diaphragm in response to a lifelong, moderate reduction in caloric intake.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health awarded to S.K. Powers (R01 HL62361) and C. Leeuwenburgh (R01 AG17994), and the American Lung Association of Florida Research Training Fellowship awarded to K.C. DeRuisseau.

ABBREVIATIONS

24AL, 24-month-old sedentary *ad libitum* fed; 24ECR, 24-month-old exercise with 8% caloric restriction; 24CR, 24-month-old sedentary with 8% caloric restriction; CAT, catalase; cDNA, complementary deoxyribonucleic acid; CR, caloric restriction; CS, citrate synthase; Cu-ZnSOD, copper-zinc superoxide dismutase; E, lifelong voluntary exercise; FAM, 6-carboxy-fluorescein; GPX, glutathione peroxidase; GSH, reduced glutathione; HO-1, heme oxygenase-1; HPRT, hypoxanthine guanine phosphoribosyl transferase; HSP, heat shock protein; LCD, liquid crystal display; Mn-SOD, manganese superoxide dismutase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SOD, superoxide dismutase; TBE, Tris-boric acid-ethylenediaminetetraacetic acid; TE, Tris-ethylenediaminetetraacetic acid.

REFERENCES

1. Baier H and Somani P. Ventilatory drive in normal man during semistarvation. *Chest* 85: 222–225, 1984.

2. Bender PR and Martin BJ. Ventilatory and treadmill endurance during acute semistarvation. *J Appl Physiol* 60: 1823–1827, 1986.
3. Criswell D, Powers S, Dodd S, Lawler J, Edwards W, Renshler K, and Grinton S. High intensity training-induced changes in skeletal muscle antioxidant enzyme activity. *Med Sci Sports Exer* 25: 1135–1140, 1993.
4. Dennery PA. Regulation and role of heme oxygenase in oxidative injury. *Curr Top Cell Reg* 36: 181–199, 2000.
5. Doekel RC Jr, Zwillich CW, Scoggin CH, Kryger M, and Weil JV. Clinical semistarvation: depression of hypoxic ventilatory response. *N Engl J Med* 295: 358–361, 1976.
6. Essig DA, Borger DR, and Jackson DA. Induction of heme oxygenase-1 (HSP32) mRNA in skeletal muscle following contractions. *Am J Physiol* 272: C59–C67, 1997.
7. Gore M, Fiebig R, Hollander J, Leeuwenburgh C, Ohno H, and Ji LL. Endurance training alters antioxidant enzyme gene expression in rat skeletal muscle. *Can J Physiol Pharmacol* 76: 1139–1145, 1998.
8. Gunduz F, Senturk UK, Kuru O, Aktekin B, and Aktekin MR. The effect of one year's swimming exercise on oxidant stress and antioxidant capacity in aged rats. *Physiol Res* 53: 171–176, 2004.
9. Hammeren J, Powers S, Lawler J, Criswell D, Martin D, Lowenthal D, and Pollock M. Exercise training-induced alterations in skeletal muscle oxidative and antioxidant enzyme activity in senescent rats. *Int J Sports Med* 13: 412–416, 1992.
10. Holloszy JO, Smith EK, Vining M, and Adams S. Effect of voluntary exercise on longevity of rats. *J Appl Physiol* 59: 826–831, 1985.
11. Itoh M, Oh-Ishi S, Hatao H, Leeuwenburgh C, Selman C, Ohno H, Kizaki T, Nakamura H, and Matsuoka T. Effects of dietary calcium restriction and acute exercise on the antioxidant enzyme system and oxidative stress in rat diaphragm. *Am J Physiol Regul Integr Comp Physiol* 287: R33–R38, 2004.
12. Ji LL. Antioxidants and oxidative stress in exercise. *Proc Soc Exp Biol Med* 222: 283–292, 1999.
13. Lass A, Sohal BH, Weindruch R, Forster MJ, and Sohal RS. Caloric restriction prevents age-associated accrual of oxidative damage to mouse skeletal muscle mitochondria. *Free Radic Biol Med* 25: 1089–1097, 1998.
14. Laughlin MH, Simpson T, Sexton WL, Brown OR, Smith JK, and Korthuis RJ. Skeletal muscle oxidative capacity, antioxidant enzymes, and exercise training. *J Appl Physiol* 68: 2337–2343, 1990.
15. Lawler JM, Powers SK, Visser T, Van Dijk H, Kordus MJ, and Ji LL. Acute exercise and skeletal muscle antioxidant and metabolic enzymes: effects of fiber type and age. *Am J Physiol* 265: R1344–R1350, 1993.
16. Leeuwenburgh C, Hansen PA, Holloszy JO, and Heinecke JW. Oxidized amino acids in the urine of aging rats: potential markers for assessing oxidative stress *in vivo. Am J Physiol Regul Integr Comp Physiol* 276: R128–R135, 1999.
17. Leeuwenburgh C, Wagner P, Holloszy JO, Sohal RS, and Heinecke JW. Caloric restriction attenuates dityrosine cross-linking of cardiac and skeletal muscle proteins in aging mice. *Arch Biochem Biophys* 346: 74–80, 1997.
18. Luhtala TA, Roecker EB, Pugh T, Feuers RJ, and Weindruch R. Dietary restriction attenuates age-related increases in rat skeletal muscle antioxidant enzyme activities. *J Gerontol* 49: B231–B238, 1994.
19. McCarter RJ, Shimokawa I, Ikeno Y, Higami Y, Hubbard GB, Yu BP, and McMahan CA. Physical activity as a factor in the action of dietary restriction on aging: effects in Fischer 344 rats. *Aging (Milano)* 9: 73–79, 1997.
20. Meister A and Anderson ME. Glutathione. *Annu Rev Biochem* 52: 711–760, 1983.
21. Navarro-Arevalo A and Sanchez-del-Pino MJ. Age- and exercise-related changes in lipid peroxidation and superoxide dismutase activity in liver and soleus muscle tissues of rats. *Mech Ageing Dev* 104: 91–102, 1998.
22. Oh-ishi S, Kizaki T, Ookawara T, Sakurai T, Izawa T, Nagata N, and Ohno H. Endurance training improves the resistance of rat diaphragm to exercise-induced oxidative stress. *Am J Respir Crit Care Med* 156: 1579–1585, 1997.
23. Oh-ishi S, Toshinai K, Kizaki T, Haga S, Fukuda K, Nagata N, and Ohno H. Effects of aging and/or training on antioxidant enzyme system in diaphragm of mice. *Respir Physiol* 105: 195–202, 1996.
24. Powers SK, Criswell D, Lawler J, Martin D, Ji LL, Herb RA, and Dudley G. Regional training-induced alterations in diaphragmatic oxidative and antioxidant enzymes. *Respir Physiol* 95: 227–237, 1994.
25. Pugh TD, Klopp RG, and Weindruch R. Controlling caloric consumption: protocols for rodents and rhesus monkeys. *Neurobiol Aging* 20: 157–165, 1999.
26. Reid MB, Haack KE, Franchek KM, Valberg PA, Kobzik L, and West MS. Reactive oxygen in skeletal muscle. I. Intracellular oxidant kinetics and fatigue *in vitro. J Appl Physiol* 73: 1797–1804, 1992.
27. Reid MB, Shoji T, Moody MR, and Entman ML. Reactive oxygen in skeletal muscle. II. Extracellular release of free radicals. *J Appl Physiol* 73: 1805–1809, 1992.
28. Selman C, McLaren JS, Collins AR, Duthie GG, and Speakman JR. Antioxidant enzyme activities, lipid peroxidation, and DNA oxidative damage: the effects of short-term voluntary wheel running. *Arch Biochem Biophys* 401: 255–261, 2002.
29. Servais S, Couturier K, Koubi H, Rouanet JL, Desplanches D, Sornay-Mayet MH, Sempore B, Lavoie JM, and Favier R. Effect of voluntary exercise on H_2O_2 release by subsarcolemmal and intermyofibrillar mitochondria. *Free Radic Biol Med* 35: 24–32, 2003.
30. Sohal RS and Weindruch R. Oxidative stress, caloric restriction, and aging. *Science* 273: 59–63, 1996.
31. Sreekumar R, Unnikrishnan J, Fu A, Nygren J, Short KR, Schimke J, Barazzoni R, and Nair KS. Effects of caloric restriction on mitochondrial function and gene transcripts in rat muscle. *Am J Physiol Endocrinol Metab* 283: E38–E43, 2002.
32. Srere P. Citrate synthase. *Methods Enzymol* 13: 3–11, 1969.
33. Starnes JW, Cantu G, Farrar RP, and Kehrer JP. Skeletal muscle lipid peroxidation in exercised and food-restricted rats during aging. *J Appl Physiol* 67: 69–75, 1989.
34. Stofan DA, Callahan LA, Di MA, Nethery DE, and Supinski GS. Modulation of release of reactive oxygen species

by the contracting diaphragm. *Am J Respir Crit Care Med* 161: 891–898, 2000.
35. Usuki F, Yasutake A, Umehara F, and Higuchi I. Beneficial effects of mild lifelong dietary restriction on skeletal muscle: prevention of age-related mitochondrial damage, morphological changes, and vulnerability to a chemical toxin. *Acta Neuropathol (Berlin)* 108: 1–9, 2004.
36. Vincent HK, Powers SK, Demirel HA, Coombes JS, and Naito H. Exercise training protects against contraction-induced lipid peroxidation in the diaphragm. *Eur J Appl Physiol Occup Physiol* 79: 268–273, 1999.
37. Vincent HK, Powers SK, Stewart DJ, Demirel HA, Shanely RA, and Naito H. Short-term exercise training improves diaphragm antioxidant capacity and endurance. *Eur J Appl Physiol* 81: 67–74, 2000.
38. Zainal TA, Oberley TD, Allison DB, Szweda LI, and Weindruch R. Caloric restriction of rhesus monkeys lowers oxidative damage in skeletal muscle. *FASEB J* 14: 1825–1836, 2000.

Address reprint requests to:
Scott K. Powers, Ph.D., Ed.D.
Center for Exercise Science
University of Florida
Rm 25 FLG
Gainesville, FL, 32611

E-mail: spowers@hhp.ufl.edu

Received after final revision October 5, 2005; accepted October 7, 2005.

This article has been cited by:

1. Samo Ribari#. 2012. Diet and Aging. *Oxidative Medicine and Cellular Longevity* **2012**, 1-20. [CrossRef]
2. Jan ŠkrhaCaloric Restriction and Oxidative Stress 83-102. [CrossRef]
3. John F Trepanowski, Robert E Canale, Kate E Marshall, Mohammad M Kabir, Richard J Bloomer. 2011. Impact of caloric and dietary restriction regimens on markers of health and longevity in humans and animals: A summary of available findings. *Nutrition Journal* **10**:1, 107. [CrossRef]
4. Alison M. Roark, Karen A. Bjorndal, Alan B. Bolten, Christiaan Leeuwenburgh. 2009. Biochemical indices as correlates of recent growth in juvenile green turtles (Chelonia mydas). *Journal of Experimental Marine Biology and Ecology* **376**:2, 59-67. [CrossRef]
5. Marwan Maalouf, Jong M. Rho, Mark P. Mattson. 2009. The neuroprotective properties of calorie restriction, the ketogenic diet, and ketone bodies. *Brain Research Reviews* **59**:2, 293-315. [CrossRef]
6. Christiaan Leeuwenburgh , Tomas A. Prolla . 2006. Genetics, Redox Signaling, Oxidative Stress, and Apoptosis in Mammalian Aging. *Antioxidants & Redox Signaling* **8**:3-4, 503-505. [Citation] [Full Text PDF] [Full Text PDF with Links]