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Archives of Biochemistry and Biophysics 401 (2002) 255–261

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Antioxidant enzyme activities, lipid peroxidation, and DNA oxidative damage: the effects of short-term voluntary wheel running

Colin Selman,[a,*] Jane S. McLaren,[a] Andrew R. Collins,[b] Garry G. Duthie,[b] and John R. Speakman[a,c]

[a] *Aberdeen Centre for Energy Expenditure and Obesity (ACERO), Department of Zoology, Aberdeen University, Aberdeen, AB24 2TZ, UK*
[b] *Division of Cellular Integrity, Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, UK*
[c] *ACERO, Division of Appetite and Energy Balance, Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, UK*

Received 8 January 2002, and in revised form 11 March 2002

Abstract

We examined the effect of voluntary exercise on antioxidant enzyme activities (catalase, glutathione peroxidase, superoxide dismutase) in skeletal muscle (hind- and forelimb) and heart of a model small mammal species: short-tailed field vole *Microtus agrestis*. In addition, DNA oxidation was determined in lymphocytes and hepatocytes using the comet assay and lipid peroxidation estimated in hindlimb muscle by measurement of thiobarbituric-acid-reactive substances. Voles ($\cong$ 6 weeks old), exposed to a 16L:8D photoperiod (lights on 0500 h), ran almost continuously during darkness. We studied the effects of voluntary running over 1 or 7 days duration, with or without an 8-h rest period, on various biomarkers of oxidative stress compared to nonrunning controls. No differences were observed in antioxidant enzyme activities, except in heart total superoxide dismutase activity ($P = 0.037$), with the lowest levels in 1- and 7-day runners at 0500 h. DNA oxidative damage, in lymphocytes or hepatocytes, and lipid peroxidation did not differ between groups. There was no evidence of any significant increase in any oxidative stress parameter in running individuals, despite having significantly elevated energy expenditures compared to sedentary controls.

Regular and moderate exercise provides various beneficial health effects, including reduced risk of cardiovascular diseases, certain cancers, osteoporosis, and obesity [35,49,55]. Even a small increase in exercise training decreases the risk of premature death in humans [15], while voluntary wheel running increased the average life expectancy of rats by almost 10% [19]. Exercise also increases oxygen consumption relative to basal levels, particularly in skeletal muscle and heart [48]. Paradoxically, this may be associated with increased production of reactive oxygen species (ROS),[1] such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$), although it has been suggested that the absence of massive oxidative damage during intense periods of cellular respiration may, in part, be due to a reduction in free radical leak across mitochondria, during state 3 respiration, at this time [10,17,26]. However, if ROS production surpasses the protection and repair mechanisms, the net effect is oxidative stress, an identified causative factor in several disease states and proposed as a factor in the cellular physiological attrition indicative of aging. Such states are primarily achieved through damage, by ROS, to macromolecules such as DNA, lipids, and proteins [7]. Exercise-induced oxidative stress has been specifically associated with disruptions in cellular homeostasis, e.g., muscle fatigue, muscle contractile dysfunction, and, after severe exercise, cellular apoptosis and muscle damage [31,37].

Perhaps this paradox is resolved by observations that many cells, including myocytes and cardiomyocytes, are capable of responding to exercise-induced increases in ROS via the induction of various repair and protection

* Corresponding author. Fax: +44-1224-272396.
E-mail address: c.selman@abdn.ac.uk (C. Selman).
URL: http://www.abdn.ac.uk/acero.

[1] *Abbreviations used:* ROS, reactive oxygen species; 8-oxodGuo, 8-hydroxy-deoxyguanosine; Cat, catalase; Gpx, glutathione peroxidase; SOD, superoxide dismutase; GSH, glutathione; TCA, trichloroacetic acid.

0003-9861/02/$ - see front matter
PII: S0003-9861(02)00050-4

mechanisms (see [51]), including enzymatic and nonenzymatic endogenous antioxidants [21,40] and stress proteins [32]. Such responses, however, are highly dependent on numerous factors including exercise type, duration, intensity, previous exercise exposure, subject age, subject species, nutritional status, tissue and fiber type examined, sampling time, and assay technique employed [20,23,27,51,54]. For example, there is conflicting evidence [24] whether acute exercise increases antioxidant levels [3,20,51]. However, it is well established that endurance training generally increases both the activities and the gene expression of several enzymatic and nonenzymatic antioxidants [3,16,18,20,22,39,41,45].

Strenuous exercise is also associated with alterations in a variety of biomarkers of oxidative stress. The ratio of urinary 8-hydroxy-deoxyguanosine (8-oxodGuo)/creatinine in marathon runners was elevated 10 h postrace, indicating that ROS generated during running may increase the DNA oxidation rate [2,44], although the finding could also be interpreted as an exercise-induced elevation in oxidative DNA repair capacity. A reduction was observed in nuclear 8-oxodGuo in rat skeletal muscle [43] after moderate swimming and in colonocytes and lymphocytes of dogs fell immediately after endurance exercise [34], suggesting an increase in DNA repair and/or antioxidant protection. In rats, forced exercise resulted in an elevation of 8-oxodGuo in heart, lung, and liver compared with those measured during spontaneous exercise and in nonexercising controls, with no differences seen between the spontaneous exercise group and controls [6]. In contrast, however, neither acute nor chronic exercise in rats altered nuclear 8-oxodGuo in several tissues [28] or in rat skeletal muscle after a single bout of exhaustive exercise, although lymphocyte 8-oxodGuo levels were elevated [53]. Estimates of lipid peroxidation tend to increase in many [9,14,24], but not all, studies [44] post-exercise bout, but the responses again appear to depend on study-specific factors [2,3]. Exhaustive exercise may however result in additional stress factors, which may potentially confound the exercise effects per se [6].

Far fewer studies have examined the effect of voluntary exercise on ROS production and oxidative stress status. A significant increase in skeletal muscle Mn-superoxide dismutase (SOD) and cytosolic glutathione peroxidase (Gpx), but not in Cu-Zn SOD or catalase, was observed in rats that had voluntarily run each day for almost 20 months compared with age-matched sedentary controls [26]. In humans, the effects of moderate exercise over 8 weeks caused no increases in muscle antioxidant status [52]. In the following study, we investigated whether short-term voluntary exercise (1 or 7 days), with or without an 8-h recovery period after a running bout, altered antioxidant enzyme activities (catalase, glutathione peroxidase, superoxide dismutase), DNA oxidation, or lipid peroxidation levels. We used a small ($\cong$ 15–30 g) microtine rodent, the short-tailed field vole *Microtus agrestis*, which ran on wheels primarily during the hours of darkness (8 h/day during this study). Antioxidant enzymes were determined in skeletal muscle (hind-and forelimb) and in heart, as oxygen consumption increases significantly in these tissues during exercise [26] and they may be particularly prone to oxidative stress due to relatively low antioxidants levels compared with other tissues [24]. Oxidative DNA damage, in lymphocytes, was measured using the modified comet assay and lesion-specific enzymes endonuclease III and formamidopyrimidine DNA glycosylase (FPG) [11–13,33] and in hepatocytes using the modified comet assay. Lipid peroxidation was measured in hindlimb muscle using the thiobarbituric-acid-reactive substances assay [5]. Using the doubly labeled water technique [50], we have found that voles with access to running wheels have daily energy expenditures over 40% higher than (same-sex sibling matched) nonrunning controls [46].

Methods

Study animals. Short-tailed field voles *M. agrestis* ($\cong$ 6 weeks old), derived from a captive breeding population at Aberdeen, UK, were maintained at 22 ± 3 °C, which is slightly outside the thermoneutral zone (25–30 °C) of this small (15–30 g) microtine rodent. Individuals were weaned at 18 days, housed individually in cages containing sawdust, given a wood chewing block, and provided with *ad libitum* water and a pelleted rodent diet (rat and mouse breeder and grower diet, Special Diets Services, BP Nutrition, UK), containing vitamin E (103.2 mg/kg), α-tocopherol (93.8 mg/kg), β-carotene (0.9 mg/kg), and vitamin C (8.0 mg/kg). Photoperiod was maintained at 16L:8D regime, with lights on at 0500 h GMT. No individual had any previous experience of a running wheel prior to this experiment and on access to a running wheel chose to run extensively, primarily during the hours of darkness (8 h). All experiments complied with a local ethical committee and passed by the UK Home Office.

Wheel running apparatus. The wheel running apparatus was designed by Dr. P. Bagley (University of Aberdeen, UK) and consisted of 40 individual cages with attached running wheels (radius 7.5 cm). Each wheel was connected to a nonconcentric disk, which on each complete rotation of the wheel operated a microswitch (RS Components, UK). The collected data was then transmitted to a PC via an optically isolated logic level converter (National Instruments, UK). Logic level data then entered the computer PCI bus via a 96-channel data acquisition card (National Instruments), of which only 40 channels were utilized during this experiment. The initiated data acquisition card then enabled information to be passed to the computer PCI bus

without processor intervention, thus allowing seamless data transmission. All software was written by P. Bagley in C (Labwindows CVI, National Instruments), allowing the data to be processed in a real time visual format, and additionally logged directly to a spreadsheet for further analysis.

Antioxidant enzyme assays. All materials used were of biochemical grade and purchased from Sigma Chemical Co. (Poole, UK). A total of 60 individuals (30 male and 30 female, 10 per group) were used to determine antioxidant enzyme activities (catalase (Cat), selenium-dependent Gpx, and total-SOD) in skeletal muscle (hind- and forelimb) and heart. Enzyme activities were measured in six experimental groups: control (no access to running wheel), 1 day (access to running wheel), or 7 days (access to running wheel), with individuals sacrificed either at 0500 h (no rest period after running bout) or at 1300 h (8-h rest period after running bout). The protocols used throughout are described fully elsewhere [47]. In brief, voles were sacrificed by cervical dislocation, and the tissues were dissected, snap-frozen in liquid N_2 within 60 s of death, and then stored at –80 °C until required. Tissue samples were subsequently thawed and homogenized in 20 vol of ice-cold 50 mM phosphate buffer (pH 7.4). The resulting homogenate was then centrifuged at 3200*g* for 20 min (5 °C), and the supernatant was used to determine antioxidant enzyme activities.

A 1% Triton X-100 treated supernatant was used to determine Cat, through the disappearance of H_2O_2 at 240 nm at 25 °C [8]. One unit of Cat represented the decrease of 1 μmol of H_2O_2 per minute. Gpx was assayed at 25 °C, with NADPH oxidation followed spectrophotometrically at 340 nm in the presence of reduced glutathione (GSH) and H_2O_2 [36]. Blanks without sample were run daily and then subtracted from actual values, to correct for spontaneous reactions in the absence of enzyme. One unit of Gpx was the amount of enzyme that oxidizes 1 μmol of NADPH per minute. Total-SOD was measured following the inhibition, at 25 °C, of pyrogallol autooxidation by SOD (with and without sample) at 420 nm [30]. One unit of total-SOD was defined as the amount of enzyme that caused a 50% inhibition of pyrogallol autooxidation. Absorbance changes were measured on a SPECTRAmax Plus microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA) using SOFTmax Pro software (Molecular Devices Corp.). Protein content was measured using the method of Lowry et al. [29], and enzyme activities were expressed per milligram protein.

Comet assay—single-cell gel electrophoresis. The level of DNA oxidative damage was determined in both lymphocytes and hepatocytes in three experimental groups all sacrificed at 1300 h: control (no access to wheel), 1 day (access to running wheel), and 7 days (access to running wheel). The comet assay, with the modification of an extra step after lysis, where DNA is digested with lesion-specific enzymes, has been described previously [11]. Plain slides were precoated in standard agarose (Gibco BRL, Paisley, UK), and isolated lymphocytes and hepatocytes were suspended in 85 μl of 1% low-melting-point agarose (Gibco BRL) and pipetted on slides. The cells were subsequently lysed by immersion in 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris–HCl (pH 10), and 1% Triton X-100 for 1 h, leaving only residual nucleoids embedded in the gel. Postlysis, slides were washed three times with enzyme buffer [0.1 M KCl, 0.5 mM Na_2EDTA, 40 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes)–KOH, 0.2 mg/ml bovine serum albumin, pH 8.0] and treated with endonuclease III or FPG. Endonuclease III converts oxidized pyrimidines to strand breaks, while FPG recognizes and breaks altered purines, thus increasing the number of breaks and the comet tail intensity. Slides were then placed in an electrophoresis tank in 0.3 M NaOH, 1 mM Na_2EDTA for 40 min unwinding time at 4 °C. After electrophoresis (30 min at 25 V and 300 mA), the slides were neutralized with Trizma base (pH 7.5) and stained with 20 μl 4,6-diamidino-2-phenylindole. Each slide was viewed by fluorescence microscopy and the degree of damage was scored visually. A total of 100 comets on each slide were assigned a score from 0 to 4, depending on the fraction of DNA pulled out into the tail. The overall score for each slide was therefore between 0 (undamaged) and 400 (completely damaged).

Thiobarbituric-acid-reactive substances. Hindlimb muscle ($n = 4$ in each group) homogenates (0.1 ml), in 0.05 M potassium phosphate (pH 7.4), were incubated at 37 °C in 2.5 ml buffer (final volume). Incubation samples were mixed with 0.5 ml 15% TCA, and peroxidation products were determined after reaction with 0.67% thiobarbituric acid. Samples were measured on a HPLC (Spheresorb 5 ODS2 (C18)), with fluorometer excitation

Table 1
Mean ± SE of the body mass (g) and the average distance run (km) for rach experimental group

	Control		1-day run		7-day run	
	1300 h	0500 h	1300 h	0500 h	1300 h	0500 h
Body mass (g)	18.3 ± 1.3	19.0 ± 2.0	20.3 ± 1.6	20.4 ± 0.9	20.1 ± 1.4	20.7 ± 0.7
Distance run (km)	—	—	4.7 ± 0.7	4.8 ± 0.8	45.1 ± 7.0	55.3 + 9.2

Table 2
Mean ± SE of catalase (Cat), glutathione peroxidase (Gpx), and total superoxide dismutase (Total-SOD) in hind- and forelimb skeletal muscle and heart in each experimental group ($n = 10$)[a]

	Control		1-day run		7-day run	
	1300 h	0500 h	1300 h	0500 h	1300 h	0500 h
Hindlimb Cat	637 ± 65	564 ± 62	628 ± 59	648 ± 75	723 ± 71	607 ± 41
Forelimb Cat	684 ± 61	497 ± 44	679 ± 59	584 ± 49	610 ± 55	645 ± 39
Heart Cat	520.1 ± 41	577.9 ± 46	655.4 ± 53	618.4 ± 42	670.0 ± 62	612.4 ± 55
Hindlimb Gpx	2.3 ± 0.16	2.3 ± 0.20	2.4 ± 0.23	2.6 ± 0.21	2.3 ± 0.20	2.7 ± 0.17
Forelimb Gpx	2.3 ± 0.20	2.3 ± 0.13	1.9 ± 0.17	2.0 ± 0.19	2.4 ± 0.21	2.0 ± 0.18
Heart Gpx	3.0 ± 0.31	3.5 ± 0.35	3.3 ± 0.38	3.4 ± 0.49	3.8 ± 0.68	3.4 ± 0.38
Hindlimb SOD	97.6 ± 13	97.3 ± 17	120.6 ± 16	85.1 ± 11	102.2 ± 16	141.1 ± 24
Forelimb SOD	98.7 ± 10	130.6 ± 19	111.2 ± 14	127.0 ± 17	138.3 ± 20	102.7 ± 8
Heart SOD	118.6 ± 10	111.3 ± 12	122.4 ± 11	91.9 ± 9	115.4 ± 10	83.5 ± 5
						$^{*}P = 0.037$

[a] All enzyme activities expressed as units min^{-1} mg protein. Differences between groups were examined by one-way ANOVA and statistical significance denoted by an asterisk.

set at 532 nm and emission at 553 nm. The results are expressed as micromoles of malonaldehyde per milligram of protein.

Statistical analyses. All values reported are means ± SE, except where indicated. Data were analyzed using SPSS (Version 9) statistical software and one-way ANOVA. Significance was indicated by P values < 0.05. Error bars in all cases denote 1 SE.

Results

The experimental groups did not differ significantly in body mass ($P = 0.81$, Table 1). The mean ± SE distance per day run by voles was 5.9 ± 1.3 km (Table 1), with voles running almost exclusively during the 8 h of darkness (lights on 0500 h GMT).

Antioxidant enzyme activities. Protein levels did not differ significantly between experimental groups in hind- ($P = 0.600$) or forelimb ($P = 0.736$) skeletal muscle or heart ($P = 0.051$). No differences were observed in the activities of Cat, Gpx, and total-SOD in either hind- or forelimb skeletal muscle between any of the experimental groups (Table 2). There were also no significant group differences in Cat and Gpx in the heart, although a significant difference ($P = 0.037$) was observed in total-SOD activity (Fig. 1, Table 2), with the lowest levels observed in 1- and 7-day runners sacrificed immediately after a running bout at 0500 h.

Oxidative DNA damage. No differences were observed in lymphocyte oxidative DNA damage between the three experimental groups (Table 3), when using either of the lesion-specific enzymes, endonuclease III ($P = 0.712$) or FPG ($P = 0.793$). No significant differences ($P = 0.726$) were seen between groups ($P = 0.726$) in hepatocyte oxidative DNA strand breaks (Table 3). In hepatocytes only, the lesion-specific enzymes did not reveal any additional oxidized bases over and above these strand breaks, in any of the three groups.

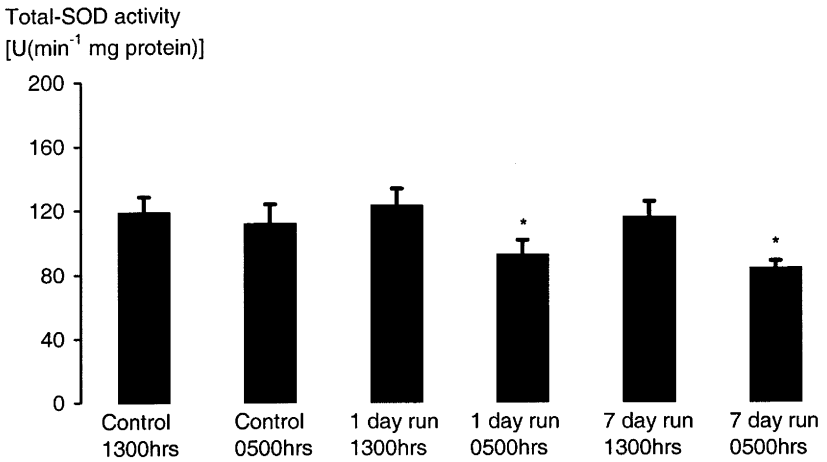

Fig. 1. Mean ± SE total superoxide dismutase activity in the heart of nonrunning control and 1- and 7-day runners sacrificed at 0500 or 1300 h. One unit of SOD is defined as the amount of enzyme that causes 50% inhibition of pyrogallol autooxidation at 420 nm. A significant difference ($P = 0.037$) was observed between the experimental groups ($n = 10$ in each group).

Table 3
Biochemical markers of oxidative stress: Mean ± SE of oxidative DNA damage (arbitrary units) using the modified comet assay and lesion-specific enzymes, endonuclease III and FPG in lymphocytes, and strand breaks in hepatocytes[a]

Biochemical marker		Control		1-day run		7 day run	
		1300 h	0500 h	1300 h	0500 h	1300 h	0500 h
Lymphocyte oxidative DNA damage	Endonuclease III $n = 6$	27 ± 12	—	46 ± 10	—	48 ± 3	—
Lymphocyte oxidative DNA damage	FPG $n = 6$	30 ± 13	—	47 ± 14	—	30 ± 6	—
Hepatocyte oxidative DNA damage	Strand breaks $n = 6$	212 ± 25	—	195 ± 22	—	243 ± 36	—
Lipid peroxidation	T-BARS $n = 4$	14 ± 2	11 ± 1	11 ± 1	13 ± 4	10 ± 1	13 ± 3

[a] Mean ± SEM of levels of lipid peroxidation (nmol malonaldehyde/mg protein), as indicated by thiobarbituric-acid-reactive substances (TBARS).

Lipid peroxidation. The levels of lipid peroxidation in the hindlimb, as estimated by measuring malonaldehyde by HPLC, did not differ significantly ($P = 0.887$) between any of the experimental groups (Table 3).

Discussion

We examined whether short-term (1- or 7-day) voluntary exercise using running wheels, with the presence or absence of an 8-h rest period, led to an alteration in various biomarkers of oxidative stress in short-tailed field voles, *M. agrestis*, compared with sedentary controls. Individual voles ran almost exclusively during the hours of darkness (8 h during this experiment). The mean distance run, at 5.9 ± 1.3 km/day, was comparable to that measured in C57/BL6 mice [4]. Previously, we have shown that access to wheels increases daily energy expenditure, estimated using doubly labeled water technique, by over 40% when compared with same-sex sibling-matched sedentary controls [46].

Endurance exercise and training lead generally to the induction of various antioxidant enzymes [25,41], although few studies have examined these changes during voluntary exercise protocols. Leeuwenburgh et al. [26] observed changes in Mn-SOD and cytosolic Gpx, but not Cat or Cu–Zn-SOD, in skeletal muscle of rats undergoing a voluntary exercise regime of nearly 20 months. However, no differences were observed in SOD, Gpx, Cat, or GSH in the skeletal muscle of humans after a moderate (35 min, three times per week) cycling program over 8 weeks [52]. It has been suggested [20] that activities of skeletal muscle antioxidants, particularly SOD, are adequate to accommodate periods of moderate oxidative stress and this may be potentially why no clear increases were observed in antioxidant enzymes in running voles compared to sedentary controls. By measuring total-SOD we do not know whether the different isoenzymes of SOD reacted differently to the exercise protocol [26]. Moderate exercise has been shown to elevate ventricular SOD activity in rats [39], although in the present study we actually observed a decrease in heart SOD activity in those individuals sacrificed immediately after either a 1- or a 7-day running bout. The reasons for this are unclear, although after exhaustive exercise, trained rats also exhibited a decrease in heart Mn-SOD when compared to controls [42].

It has been suggested that exercise intensity is an important factor in DNA oxidative damage, with spontaneous activity actually maintaining a low level of DNA oxidative damage, although we found no evidence of this in our study, using the comet assay and lesion-specific enzymes. The response of various tissues to exercise-induced oxidative DNA damage may also differ [53]. For example, the levels of oxidative DNA damage in rats, as indicated by heart, lung, and liver 8-oxodGuo, were shown to increase during forced exercise compared to control levels, but the levels after spontaneous exercise did not [6]. Moderate swimming actually reduced skeletal muscle nuclear 8-oxodGuo [43], with neither acute nor chronic exercise in rats causing any variation in nuclear 8-oxodGuo in a variety of tissues [28]. An exercise-induced increase in ROS has been implicated in many [9,14], but not all [3], studies that have used exhaustive or endurance exercise protocols, although we observed no evidence of increased lipid peroxidation in runners compared to controls in the present study.

A considerable body of work exists on the consequences of various exercise regimes on oxidative stress parameters (for review see [41]) but very few have examined these parameters using a model of voluntary exercise [26,52]. This is the first study in rodents, to our knowledge, that has examined the relationship between measured increases in metabolism during voluntary exercise and various measures of oxidative stress, although similar study protocols have been studied in humans (e.g., [14]). We have shown that despite wheel running increasing daily energy expenditure by over 40% compared to sedentary controls [46], this did not elicit any clear increases in antioxidant enzyme activities, oxidative DNA damage, or lipid peroxidation. In contrast, a previous study, using the same model showed that long-term cold exposure (8 ± 3 °C) elevated the activities of Cat in skeletal muscle (gastronemius), kidney, and heart

and Gpx in the heart in short-tailed field voles compared to control animals kept at $22 \pm 3\,^{\circ}C$ [47]. Therefore, it would appear that in this study, the antioxidant protection and repair mechanisms were sufficient to cope with any increases in ROS or that the duration [26] and/or intensity of exercise were insufficient to induce significant and measurable increases in oxidative stress in this mammalian model. This is analogous to the U-shaped curve relationship between the duration and intensity of exercise and oxidative modification of DNA suggested by Poulsen et al. [38]. However, the risk of oxidative damage, particularly in muscle, during exercise may also be reduced because free radical leak across the mitochondrial membrane may be lower at this time (e.g., [17,26]). In addition, exercise of a forced and endurance nature may also result in additional stressors [1,6], where it may be difficult to isolate the effects solely attributable to exercise-induced oxidative stress, compared to those due to other complicating factors. Short-term voluntary exercise protocols, as in our study, may help minimize these additional, and potentially confounding, stress factors.

Acknowledgments

This work was supported, in part, by a grant from the Biotechnology and Biological Sciences Research Council (SAGE-1 initiative) to J.R.S. We are grateful to the animal house staff (Duncan Wood, Shona Fleming, and Jim Levenie), to Suzanne Lumsden, and to Dr. Phil Bagley for designing and installing the wheel running apparatus (all University of Aberdeen, UK). C.S. thanks Professor G. Barja for the antioxidant enzyme protocols and J.M. is grateful to Nik Vaughan, Sharon Wood, Sabrina Narayanan, and Philip Morrice at the Rowett Research Institute, UK, for help and advice. All experiments complied with the current laws of the UK and were carried out under license from the UK Home Office.

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