Oxidative Stress Response in Normal and Antioxidant Supplemented Rats to a Downhill Run: Changes in Blood and Skeletal Muscles

Tongjian You1, Allan H. Goldfarb2, Richard J. Bloomer3, Linh Nguyen2, Xin Sha4, and Michael J. McKenzie2

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Abstract/Résumé
The purpose of this study was to determine if changes in oxidative stress biomarkers in blood and skeletal muscles are similar in normal and antioxidant supplemented rats after a downhill run. Sixty-six male Sprague-Dawley rats were pretreated with a normal rat diet or diet + antioxidants (2,000 mg vitamin C + 1,000 IU vitamin E/kg diet) for 2 weeks. Exercised rats ran 90 min on a rodent treadmill at a speed of 16 m/min at −16° grade. Rats were sacrificed either at rest, immediately, 2 hrs, or 48 hrs postexercise. Malondialdehyde (MDA) and protein carbonyl (PC) concentrations and glutathione status in blood, vastus lateralis (white fast-twitch), vastus intermedius (red fast-twitch), and soleus (slow-twitch) muscles were determined. A significant increase from rest in PC occurred in plasma, vastus intermedius and soleus muscle 2 hrs after the downhill run (p < 0.05), with no changes observed at any other times postexercise. Antioxidant supplementation significantly decreased PC concentrations in both vastus intermedius and soleus muscles at all times combined (p < 0.05).

1Gerontology and Geriatric Medicine, Wake Forest U. School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157; 2Dept. of Exercise and Sport Science, University of North Carolina at Greensboro, Greensboro, NC 27402; 3Dept. of Health and Sport Sciences, University of Memphis, Memphis, TN 38152; 4Dept. of Chemistry, Wake Forest University, Winston-Salem, NC 27109, USA.
MDA and glutathione status in blood and muscles were unaffected by either the downhill run or antioxidant treatment. For PC and MDA, the concentrations were lower in blood as compared to skeletal muscle, with the opposite finding for oxidized glutathione; however, the pattern of response postexercise was similar. These data indicate that (a) PC, but not MDA or oxidized glutathione, is elevated transiently following downhill running in male rats; (b) the elevation in PC postexercise occurs in plasma, vastus intermedius, and soleus muscles; (c) antioxidant therapy can attenuate PC in vastus intermedius, and soleus muscles; and (d) while the concentrations of oxidative stress biomarkers differ between blood and the various skeletal muscles, the pattern of response postexercise is similar.

Introduction

Eccentric muscle actions can cause mechanical trauma to the skeletal muscle fibers (Armstrong, 1984). Typically, eccentric muscle actions result in muscle soreness, microfiber insult, loss of muscle strength, decreased range of motion, and a progressive secondary inflammatory response to the muscle (Armstrong, 1984). The secondary inflammatory process results in macrophage and neutrophil infiltration into the muscle and produces reactive oxygen species (ROS) and other cytotoxic factors. These ROS can help to degrade muscle proteins but may also result in further damage. Eccentric exercise-induced oxidative damage has been examined in several animal and human studies (Kanter et al., 1993; Lee et al.,
The results from these studies are inconsistent, in part because of the measures of oxidative stress and the type of exercise.

Dietary antioxidants have been used as a preventive treatment for oxidative stress and muscle damage and also as a therapy for repair processes (Goldfarb, 1999). A growing number of studies have examined the effect of antioxidant supplementation on aerobic concentric exercise-induced oxidative stress (Alessio and Goldfarb, 1997; Faff and Frankiewicz-Jozko, 1997; Hartmann et al., 1995; Kaikkonen et al., 1998; Reznick et al., 1992; Rokitzki et al., 1994a; 1994b; Sen et al., 1997; Sumida et al., 1997; Thompson et al., 2001), and most of them reported a beneficial effect. Goldfarb et al. (1994) measured oxidative stress biomarkers in three muscle groups—vastus lateralis, vastus intermedius, and soleus—in normal and antioxidant supplemented rats. They examined oxidative stress in response to aerobic treadmill running and noted an increase in lipid peroxidation in the muscles examined. They also reported that vitamin E supplementation could attenuate the oxidative stress in certain muscles.

However, the antioxidant supplementation effect on eccentric exercise-induced oxidative damage has not been well studied, especially in different muscle fiber types. In a study on humans, Meydani et al. (1993) reported that 48 days of vitamin E supplementation reduced muscle conjugated dienes, a lipid peroxidation marker, following a 45-min downhill run. In contrast, Warren et al. (1992) reported no protection from vitamin E supplementation in soleus muscles to oxidative stress in rats following a 150-min downhill run. However, both studies only measured one biomarker in the muscles.

Previous studies have reported that slow-twitch muscles have higher basal oxidative stress and greater antioxidant capacity compared to fast-twitch muscles (Ji et al., 1992; Goldfarb et al., 1994). Therefore, muscles composed of different fiber types probably have different responses to exercise and antioxidant supplementation. In addition, an increase in production and release of oxidative products from these muscles may alter the levels of oxidative stress biomarkers in the circulation. However, it is unknown whether the oxidative stress response following eccentric exercise is similar in blood and different skeletal muscles.

Thus, the purpose of this study was to compare the oxidative stress response in blood and skeletal muscles in normal diet and antioxidant-supplemented diet for 2 weeks in rats after a downhill run. The hypotheses were as follows: (1) downhill running would result in oxidative stress, as indicated by an increase in at least one of the biomarkers (MDA, PC, and glutathione status) in the blood and skeletal muscles; (2) antioxidant supplementation would reduce the extent of oxidative stress as compared to the normal diet; and (3) the relative changes in oxidative stress biomarkers in blood and different skeletal muscles would follow a similar pattern in response to downhill running and antioxidant supplementation.

**Methods**

**ANIMALS, PROTOCOLS, AND SAMPLING**

Sixty-six young male Sprague-Dawley rats (6–8 weeks old, 142–205 g) were purchased from Harlen-Teklad Company. All procedures for animal care and use were approved and filed with the university’s animal review committee prior to the study.
The rats were housed 2 per cage in a temperature (21 ± 1 °C) and humidity (35 ± 5%) controlled environment with free access to food and water. Food intake and body weight of each rat were recorded daily. The rats were housed for 1 week prior to receiving any treatment. During this period all rats were accustomed to treadmill running at a speed of 10–15 m/min at a grade of 0° on a rodent treadmill for 15 min.

The rats were randomly assigned to either a normal diet (N) or antioxidant diet (A) treatment. The N diet consisted of standard rat chow supplied by the Harlen-Teklad Company. This diet contained 0 mg Vitamin C and 153 IU vitamin E. The A treatment had their diet supplemented with 2,000 mg vitamin C and 1,000 IU vitamin E (α-tocopheryl acetate) per kg diet. This dose was chosen because it is within the safe range and would be similar to diets that have been used for both of these antioxidants separately in studies on rats (Gohil et al., 1986; Goldfarb et al., 1994; McIntosh et al., 1993; Reznick et al., 1992). The diet supplementation period was for 2 weeks prior to the exercise and until the rats were killed.

After 2 weeks of diet supplementation the rats were further randomly divided into a control rested group (R = 18) and an exercised group (E = 48). The rats that were exercised ran 90 minutes on a rodent treadmill at a speed of 16 m/min and a grade of −16°. This type of exercise has been reported to result in muscle damage in rats (Armstrong et al., 1983). Rats from both the N and A diet treatments were killed at rest, or immediately, 2 hours, or 48 hours after exercise.

Rats were killed by decapitation, and mixed blood was collected into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). One milliliter of whole blood was treated immediately for glutathione analysis. The remainder of the blood was centrifuged at 4 °C at 3,000 rpm for 20 minutes. Plasma was stored in a −80 °C freezer until subsequent analyses of plasma protein, MDA, and PC. Vastus lateralis (superficial, mainly white fast-twitch, type IIx), vastus intermedius (deep, mainly red fast-twitch, type IIa), and soleus (mainly slow-twitch, type I) muscles of both legs were quickly obtained and fast frozen on dry ice. Tissues were then transferred to a −80 °C freezer until analyses of muscle protein, MDA, PC, and glutathione status.

**BIOCHEMISTRY ANALYSES**

**Protein Concentrations.** Plasma and muscle protein concentrations were determined by the Lowry method (Sigma Co. P5656). Muscle samples were weighed and homogenized with a motor-driven homogenizer at 0 to 4 °C in PBS, pH 7.4. The homogenates were centrifuged at 11,000 × g for 10 min at 4 °C. The supernatants were analyzed for muscle protein concentrations. PC and MDA concentrations were adjusted to amount of protein. Protein concentrations and all the oxidative stress biomarkers were determined in duplicate.

**MDA Concentrations.** Plasma and muscle MDA concentrations were analyzed by a colorimetric method reported by Gerard-Monnier et al. (1998) with a modification by using 1,1,3,3-tetramethoxypropane as MDA standard. Plasma samples are directly used for this assay. Muscle samples were washed with 10 mM PBS, pH 7.4, weighed, and then homogenized in 10 mM PBS containing 4 mM BHT. The homogenates were centrifuged and the supernatants were analyzed for total MDA.
Initially, 500 mM butylated hydroxytoluene (BHT) in acetonitrile (11 µl), standard or sample (210 µl), and 12 N hydrochloric acid (HCl) (5.3 µl) were added to each tube and mixed well. For each plasma or muscle sample there was a test tube and a blank tube. The tubes were incubated at 60 °C for 80 minutes. After the tubes were cooled to room temperature, working reagent (680 µl) was added to the standard and sample test tubes. Instead of the working solution, an acetonitrile/methanol (3:1, v/v) solution (680 µl) was added to the sample blank tube. The tubes were mixed and centrifuged, and the supernatant (660 µl) was collected and then 12 N HCl (115 µl) was added to each tube and mixed well. The tubes were further incubated at 45 °C for 60 min. After incubation, the tubes were further centrifuged. The supernatant (700 µl) was collected, and the absorbance was measured at 586 nm in a Shimadzu spectrophotometer. The OD value for the blank tube was subtracted from the test tube value. The data were plotted for MDA concentrations vs. O.D. values and compared to the standard curve.

**PC Concentrations.** Plasma and muscle PC concentrations were determined via the method of Reznick and Packer (1994), monitoring the amount of 2, 4-dinitrophenylhydrazine (DNPH) bound spectrophotometrically at 360 nm. Plasma samples were directly used. Frozen muscle samples were weighed and placed in 50-mM potassium phosphate buffer (pH 7.4) containing 0.1% Triton X and protease inhibitors leupeptin (1.0 µg/ml), pepstatin (1.4 µg/ml), and aprotinin (1.0 µg/ml). Muscle samples were homogenized at 0 to 4 °C at 500 rpm. The homogenates were centrifuged, and the supernatant (900 ul) and 10% streptomycin sulfate in 50-mM potassium phosphate buffer (100 ul) were mixed and incubated at room temperature for 15 min. The samples were centrifuged and the supernatants were used for determination of PC concentrations.

Samples (125 ml) were added to two test tubes. One sample was mixed with 10 mM DNPH in 2.5 N HCl (500 ul) while the other sample was mixed with 2.5 N HCl (500 ul). The samples were incubated 1 hour at room temperature in the dark and were vortexed every 15 min. Then 20% trichloroacetic acid (TCA) (625 ul) solution was added in the tubes and left on ice for 10 min and then centrifuged for 5 min to obtain precipitates. The pellets were washed once more with 10% TCA (1 ml) and were broken with the aid of a glass rod. Then the pellets were washed 3 times with 1 ml of ethanol-ethyl acetate (1:1) (v/v). The precipitates were dissolved in 1 ml of 6 M guanidine HCl solution containing 20 mM potassium phosphate (pH 2.3). The tubes were vortexed and then placed into a 37 °C water bath for 30 min. Insoluble materials were then removed by centrifugation. The solutions were collected and the absorbance was measured at 361 nm in the spectrophotometer. Each sample was read against the sample treated with 2.5 N HCl. Protein carbonyl concentration was calculated by using a molar absorption coefficient of 22,000/M/cm.

**Glutathione Status.** Blood and muscle glutathione status was analyzed by the DTNB-GSSG reductase recycling method (Anderson, 1985). Blood samples were processed immediately for preparation of total glutathione (TGSH) and oxidized glutathione (GSSG). For blood TGSH, whole blood (500 µl) and 10% 5-sulfosalicylic acid containing 1 mM bathophenantrolinedisulfonic acid (BPDS) (1 ml) were mixed and then centrifuged twice at 11,000 x g for 10 min at 4 °C. The supernatant was collected and stored at –80 °C. For blood GSSG, whole blood
(500 µl), and 10% 5-sulfosalicylic acid containing 1 mM BPDS (250 µl) were mixed and then centrifuged twice at 11,000 x g for 10 min at 4 °C. The supernatant (300 µl) was collected and mixed with 2-vinylpyridine (6 µl). Triethanolamine (18 µl) was added into the tube and the pH was adjusted between 6.0–7.0 by adding 1 N HCl and monitoring this on a Corning pH meter. The tubes were vortexed again and then allowed to incubate at room temperature for 1 hour. The samples were then stored in a –80 °C freezer until analyzed.

Muscle samples were weighed and placed in 5% 5-sulfosalicylic acid containing 1 mM bathophenanthroline disulfonic acid (BPDS). The samples were homogenized with a motor-driven homogenizer at 0 to 4 °C and then centrifuged at 4 °C. The supernatants were analyzed for muscle total glutathione (TGSH) and oxidized glutathione (GSSG) concentrations.

For TGSH assay, standards were made from GSH (1 mM) and diluted daily with 10% 5-sulfosalicylic acid containing 1 mM BPDS. First the standard (25 µl) or sample (25 µl) was added into a cuvette. Then 0.3 mM NADPH (700 µl), 6 mM DTNB (5,5-dithio-bis-2-nitrobenzoic acid) (100 µl), and distilled water (175 µl) were added, mixed, and equilibrated for 12–15 minutes at 30 °C. After equilibration, GSSG reductase (266 u/ml, 10 ml) was added into the cuvette and read at 412 nm in the spectrophotometer for 66 seconds. The change in the absorbance was plotted vs. time slope to get a determination of concentration in the cuvette. The data was plotted for the concentration vs. the slope and compared to the standard curve. The TGSH concentration read from the standard curve was multiplied by the dilution factor to obtain the sample TGSH concentration. The procedures for GSSG assay were the same as those for TGSH assay except for using GSSG as standards and multiplying a different dilution factor. Oxidized glutathione (GSH) concentration was calculated by subtracting GSSG from TGSH. Glutathione status was expressed in (GSSG/TGSH)*100.

**STATISTICAL ANALYSIS**

Statistical analysis for this study used a JMP 4.0 statistical software package (SAS Institute, Cary, NC). The data were analyzed by a two-factor (4 × 2) ANOVA examining downhill exercise and antioxidant supplementation. A Tukey HSD post hoc test was used in order to ascertain where differences might be. A student \( t \)-test was used to compare animal weight, run time, and oxidative stress at any time point between the two treatment groups. Significance was set at the 0.05 level. Data are presented as means ± SE.

**Results**

**ANIMAL WEIGHT**

The initial mean weights of the rats after random assignment were 167.5 ± 2.5 g and 169.7 ± 2.6 g in the N and A treatments, respectively, and were not statistically different. The weight of each animal increased in a normal manner during the entire study. The final mean weights on the day when rats were killed were 292.4 ± 3.0 g and 298.8 ± 3.1 g in the N and A treatments, respectively. There was no significant treatment effect on final animal weight.
A total of 48 rats ran on the treadmill. However, 4 animals incurred foot injuries and were not included in the data analyses. Thirty-eight animals (86.36%) ran the entire 90 minutes while the other 6 ran between 47 to 89 min (4 from the N and 2 from the A treatments). All 44 rats without injuries were included in the analyses since they completed at least half of the 90-min run. The mean run time of the exercised animals in the two treatments is shown in Figure 1. There was no significant between-treatment difference in run time.

**MDA AND PC CONCENTRATIONS**

Plasma and muscle MDA concentrations for each treatment at each time point are presented in Figure 2. There was no significant time, treatment, or time and treatment interaction effect for MDA in plasma or in the three muscle fiber types.

Plasma and muscle PC concentrations for each treatment at each time point are shown in Figure 3. A significant effect of time, but not treatment or time and treatment interaction, was found for plasma PC concentrations. The HSD post hoc test indicated that plasma PC significantly increased from $0.67 \pm 0.05 \text{ nmol/mg protein}$ at rest to $0.91 \pm 0.04 \text{ nmol/mg protein}$ ($p < 0.05$) at 2 hours after the downhill run when the two treatment groups were combined. There was no significant time, treatment, or time and treatment interaction effect on PC in vastus lateralis. A significant main effect of time and treatment, but not time and treatment interaction, was found in vastus intermedius and soleus muscles. The HSD post hoc test indicated that PC significantly increased from $2.46 \pm 0.16 \text{ nmol/mg protein}$ at rest to $3.35 \pm 0.24 \text{ nmol/mg protein}$ at 2 hrs after the run in the vastus intermedius ($p < 0.05$), and from $3.04 \pm 0.22 \text{ nmol/mg protein}$ at rest to $4.22 \pm 0.33 \text{ nmol/mg protein}$ at 2 hrs in the soleus ($p < 0.05$) when the two treatment groups were combined. The treatment main effect noted that the A treatment had significantly lower PC compared to the N treatment in both the vastus intermedius and the soleus muscles ($p < 0.05$). The between-treatment difference at 48 hrs tended to be significant in soleus muscle ($p = 0.06$).
Figure 2. Malondialdehyde (MDA) concentrations in plasma and muscles of rat hindlimb (A: plasma; B: vastus lateralis; C: vastus intermedius; D: soleus) before exercise (BE), and 0 hr, 2 hrs, and 48 hrs postexercise (0 PE, 2 PE, 48 PE, respectively) in the normal diet (○) and antioxidant diet (■) treatments, 7–9 animals per group at each time point. No statistical difference across time or between treatments for plasma or muscles.

GLUTATHIONE STATUS

Blood glutathione status was measured in all animals. However, muscle glutathione status was only determined in the vastus muscles (3–7 animals per treatment at each time point) since there was inadequate muscle tissue from the soleus. The results indicated that there was no significant time, treatment, or time and treatment interaction effect on TGSH, GSH, GSSG, and GSSG/TGSH ratio in blood, vastus lateralis, or vastus intermedius. The ratio of GSSG/TGSH for blood and the vastus muscles is presented in Figure 4.

Discussion

This study compared the changes in several oxidative stress biomarkers, including MDA, PC, and glutathione status, in blood and in different rat hindlimb muscles in response to downhill running and dietary antioxidant pretreatment (vitamins C and E). We found that PC concentrations were elevated in plasma, vastus intermedius, and soleus muscles after the run, and that antioxidant supplementation reduced this oxidative stress marker in these muscles. In addition, MDA and glutathione status in the blood and muscles were unaffected by the downhill run or the antioxidant treatment. Thus the response appears to differ based on the biomarker
Figure 3. Protein carbonyl (PC) concentrations in plasma and muscles of rat hindlimb (A: plasma; B: vastus lateralis; C: vastus intermedius; D: soleus) before exercise (BE), and 0 hr, 2 hrs, and 48 hrs postexercise (0 PE, 2 PE, 48 PE, respectively) in the normal diet (■) and antioxidant diet (▲) treatments, 7–9 animals per group at each time point. Significant time effect was found in A, C, and D; a: \( p < 0.05 \), compared with BE (2 treatment groups combined). Significant treatment effect was found in C and D at all times combined; b: \( p < 0.05 \), main treatment effect; c: \( p = 0.06 \), compared with antioxidant diet at 48 PE.

studied, as well as the tissue used for analysis. Additionally, while the concentrations of oxidative stress biomarkers differed between blood and the various skeletal muscles, the pattern of response postexercise was similar.

During the phagocytic stage of muscle damage, neutrophils, macrophages, and other inflammatory cells may accumulate in the muscle tissues. Inflammatory cell accumulation has been reported to appear within 1–6 hrs post muscle injury. These cells phagocytize tissue debris and release cytotoxic factors such as superoxide radicals and proteases that contribute to protein degradation and oxidative stress (Tidball, 1995). In the present study, the downhill run resulted in an increase in PC concentrations at 2 hrs after exercise in the plasma and in the vastus intermedius and soleus muscles. These results suggest that inflammatory reactions may partially contribute to the increase in PC observed as early as 2 hrs after exercise. In a recent human study, Lee et al. (2002) reported an increase in plasma PC at 24 and 48 hrs after eccentric elbow flexion exercise in males. Our results suggest that the PC increases in the plasma and hindlimb muscles of rats occurred at an earlier
time than what has previously been reported, but that it returns to resting values sooner rather than after high force eccentric exercise as reported by Lee et al. (2002). However, human and animals studies are different and the exercise models are not entirely comparable.

Previous animal studies have found that an exhaustive treadmill run resulted in an increase in hindlimb muscle TBARS at 3 hrs after exercise in untrained rats (Faff and Frankiewicz-Jozko, 1997; Frankiewicz-Jozko et al., 1996). The increased TBARS values were explained by the possible inflammatory response related to the muscle damage. The commonly used TBARS assay has been suggested to overestimate the actual MDA values due to its reactivity with other aldehydes, sugars, amino acids, and bilirubin (Meagher and FitzGerald, 2000). In the present study, MDA values were not significantly altered but PC values were significantly increased in both plasma and muscle at 2 hrs after exercise. Based on these results, amino acid oxidation could be a reasonable explanation for the increase in TBARS reported by these other studies. This also suggests that proteins may be more sus-

Figure 4. Glutathione status (GSSG/TGSH*100) in whole blood and muscles of rat hindlimb (A: whole blood; B: vastus lateralis; C: vastus intermedius) before exercise (BE), and 0 hr, 2 hrs, and 48 hrs postexercise (0 PE, 2 PE, 48 PE, respectively) in the normal diet (□) and antioxidant diet (■) treatments, 3–9 animals per group at each time point. No statistical difference across time or between treatments for blood or muscles.
ceptible to breakdown by proteases and then subsequent oxidation by ROS compared to lipid peroxidation in response to this type of exercise stress.

The present study reports that the downhill run did not significantly change glutathione status in blood and the vastus muscles. These results did not support the findings of Best et al. (1999), which indicated that there was an increase in total and reduced glutathione in muscle after severe mechanical stretch of the ankle flexor in rabbits. In the present study it is possible that the alteration of glutathione status was not significant enough in the tissues being examined, due to the relative low intensity of eccentric exercise.

This was the first study to investigate antioxidant treatment and oxidative stress in blood and different skeletal muscle fiber types following eccentric exercise. Confounding results have been reported by current studies regarding antioxidant effect on oxidative stress following eccentric exercise. Vitamin E pretreatment did not affect soleus muscle resistance to pro-oxidants in female rats (Warren et al., 1992); however, only one marker was measured in one hindlimb muscle. In addition, combined antioxidant treatment and oxidative stress have not been well investigated during eccentric exercise. The present study indicates a mild protective effect of combined antioxidant dietary treatment in the hindlimb muscles in response to the downhill run.

It is possible that humans might have a different response since rodents can produce vitamin C, which may be sufficient for their normal oxidative stress control at rest. However, this antioxidant capability may not be enough to control and quench excessive ROS production induced by eccentric exercise. The results from the present study suggest that the antioxidant defense in these muscles was insufficient to protect the proteins from oxidation in response to the downhill run. The addition of this combined antioxidant diet was mildly successful in protecting these muscles from protein oxidation.

Our results showed that MDA and PC concentrations in plasma were lower than those in the muscles. Among the different muscles, MDA and PC concentrations were highest in the soleus and lowest in the vastus lateralis. These results agree with the findings of Goldfarb et al. (1994) on TBARS in these muscles and antioxidant enzymes. The differences are probably due to the differences in capillary density, mitochondrial content, and oxidative capacity in these muscles. Taken together, our data supports the contention that the more aerobic muscles have higher levels of oxidative stress biomarkers than the less aerobic muscles. However, in the present study, oxidized glutathione was actually slightly higher in the vastus lateralis compared to the vastus intermedius muscle, and changes in blood glutathione status did not follow those in the muscles. This could be explained by two reasons. First, the glutathione in the blood can be influenced by the liver. Second, the blood glutathione system may not be as sensitive to acute changes as muscle. But as noted, the intensity of this exercise was fairly modest and thus the glutathione system may not have been stressed sufficiently.

There are some limitations in the current study. First, since we did not include a level runner group in our design, it is difficult to distinguish the effect of concentric exercise from this downhill running. Second, our downhill exercise model was based on previously published work, and muscle damage was not confirmed in our study. Third, sample sizes for certain markers, such as glutathione status, are relatively small and may lower the statistical power.
In summary, the present study demonstrates that 90 minutes of downhill running could induce oxidative stress as indicated by increases in PC in plasma, vastus intermedius and soleus, but not vastus lateralis muscles, at 2 hours after exercise, with little change in MDA or glutathione status at any time postexercise. Two weeks of antioxidant pretreatment significantly decreased PC in the vastus intermedius and soleus muscles after the downhill run. This suggests that this antioxidant pretreatment can partially protect these tissues from eccentrically induced oxidative stress. In addition, while the concentrations of oxidative stress biomarkers differ between blood and the various skeletal muscles, the pattern of response postexercise is similar.

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