No evidence of oxidant stress during high-intensity rowing training

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DERNBACH, A. R., W. M. SHERMAN, J. C. SIMONSEN, K. M. FLOWERS, AND D. R. LAMB. No evidence of oxidant stress during high-intensity rowing training. J. Appl. Physiol. 74(5): 2140–2145, 1993.—In untrained subjects, strenuous exercise provokes the appearance of oxidant stress markers in blood and muscle. On the other hand, trained muscle is resistant to oxidant stress unless exercise challenges the muscle glycogen supply. It is not known whether chronic high-intensity exercise alters the susceptibility of skeletal muscle to oxidant stress, whether there are gender-related differences in markers of oxidant stress, or whether elevating muscle glycogen stores by increasing dietary carbohydrate can minimize any exercise-related oxidant stress. To address these issues, collegiate rowers (12 men, 11 women) were randomly assigned to a moderate- (MOD, 5 g/kg body wt) or high-carbohydrate (HI, 10 g/kg) diet in a double-blind design and underwent strenuous training for 4 wk. Training in the A.M. was 40 min at 70% maximal \( V_{\text{O}2} \) consumption (\( V_{\text{O}2}\)); in the P.M. it was either three 2,500-m time trials (to assess power output) or aerobic and lactate tolerance training. Total daily training time was 65 min at 70% maximal \( V_{\text{O}2} \), and 38 min at \( \geq 90\% \) maximal \( V_{\text{O}2} \). Three-weekly morning blood samples were assayed for serum creatine kinase (CK), plasma thiobarbituric acid-reactive substances (TBARS), and serum \( \beta \)-glucuronidase (\( \beta \)-Gluc). Weekly muscle biopsies were obtained for analysis of glycogen and, when tissue sample quantity allowed, TBARS. HI rowers produced more power and improved power more (10.7 ± 1.0 vs. 1.6 ± 1.6%) over the 4 wk than did the MOD rowers. Preexercise muscle glycogen concentration was maintained at 119 mmol/kg in MOD but increased 65% in HI rowers (\( P < 0.05 \)). Main effects of time were observed for plasma TBARS, CK, and \( \beta \)-Gluc (\( P < 0.05 \)), but pairwise post hoc comparisons of means at different times were not significant. CK values averaged 55% lower in female than in male rowers (\( P < 0.05 \)), but there were no gender-related effects on the CK response to training. Sex-by-treatment interaction (\( P < 0.05 \)) indicated that the plasma TBARS response was higher in the male HI rowers than in the other groups. It was concluded that the training caused no apparent oxidant stress/muscle damage and that both men and women rowers on either diet were able to adapt to the imposed training load.

oxidant stress; exertion; thiobarbituric acid-reactive substances

EXERCISE MAY IMPOSE an oxidant stress on muscle tissue (1, 11, 14), causing free radical formation that is at least partly attributed to the high utilization of \( O_2 \) by contracting muscle (13, 33). Damage to mitochondria, sarcoplasmic reticulum, and other components of skeletal muscle is associated with a variety of modes and intensities of exercise in both laboratory animals and humans (1, 2, 11, 14, 22, 28). The muscle damage has often been related to the attack of free radicals on the polyunsaturated fatty acids of cellular membranes, resulting in lipid peroxidation and/or protein degradation (24, 28, 29, 34). Oxidant stress has not been investigated in subjects engaged in chronic high-intensity exercise training. Accordingly, one purpose of this study was to determine whether high-intensity twice-daily rowing training would affect selected markers of oxidant stress.

A second purpose of the experiment was to characterize possible gender-related differences in exercise-induced oxidant stress. The rationale for this aspect of the investigation is that greater concentrations of circulating estrogens in females may reduce oxidant stress in membranes (25, 36). For example, mitochondrial and microsomal fractions from skeletal muscle are less susceptible to peroxidation in female than in male mice (21). Also, creatine kinase (CK) leakage from muscle of treadmill-exercised rats is highly elevated (67%) in males but only moderately increased (114%) (4) or unchanged in females unless they are ovarioctomized (3). Bar et al. (7) also reported that both male rats pretreated with an estrogen compound and ovarioctomized female rats treated with estrogen replacement showed no exercise-induced muscle damage. Finally, 2 h of cycle ergometry at 50% maximal \( O_2 \) consumption (\( V_{\text{O}2} \)) caused a 47% increase in plasma CK activity 24 h after exercise in men but only a 9% increase in women (8).

The third purpose of the study was to determine whether routine consumption of a high-carbohydrate diet during strenuous training might enhance muscle glycogen stores and thereby minimize markers of oxidant stress during training. Exercise-trained skeletal muscle, otherwise resistant to oxidant stress (1, 19, 28, 35), may be more susceptible to lipid peroxidation if dietary carbohydrate is insufficient to maintain high concentrations of glycogen in muscle (20, 22). High-intensity exercise training places a substantial demand on glycogen stores in active skeletal muscle and sometimes results in significant acute and chronic glycogen depletion (29); this glycogen must be replenished by dietary carbohydrate between training sessions (9). When large amounts of dietary carbohydrate are consumed, glycogen stores are increased, and the ability to perform high-intensity work is enhanced (9). Accordingly, it is possible that lipid peroxidation occurring in skeletal muscle during prolonged exercise (12, 23) might have been minimized if a high-
carbohydrate diet had improved the availability of glucose for the hexose monophosphate shunt. Greater availability of substrate for the shunt might decrease the potential for lipid peroxidation by maintaining or increasing the production of NADPH and thus increasing the reducing potential of the cell. Therefore it was hypothesized that athletes undergoing chronic high-intensity training with a large intake of dietary carbohydrate would exhibit greater muscle glycogen stores and decreased values for markers of oxidant stress, muscle damage, and/or increased membrane permeability compared with athletes undertaking the same training while consuming a moderate-carbohydrate diet. The power output and muscle glycogen results have been reported in detail (30).

METHODS

Characteristics of subjects and diets, protocols for training and peak \( \dot{V}O_2 \) testing, and techniques for muscle biopsy have been described elsewhere (30). Collegiate rowers (12 men, 11 women) volunteered to serve as subjects and provided informed consent according to institutional guidelines. Rowers were chosen as subjects because their average maximal \( \dot{V}O_2 \) and potential for free radical formation are high relative to other athletes. Rowers also routinely train at exercise intensities requiring a high percentage of maximal \( \dot{V}O_2 \) (16).

**Diet.** The rowers were randomly assigned, in a double-blind design, to a high-carbohydrate (HI) group (10 g carbohydrate/kg body wt) or a moderate-carbohydrate (MOD) group (5 g carbohydrate/kg body wt). The diets were similar, except the HI diet included a liquid glucose polymer supplement (Exceed Hi-Carb, Ross Laboratories, Columbus, OH) that supplied ~29 and 41% of daily dietary energy and carbohydrate, respectively. The MOD diet included an artificially colored, flavored, and textured liquid placebo.

**Training protocol.** The subjects undertook 4 wk of 6-day/wk twice-daily training. The morning workout included cycling on a Monark cycle ergometer (weeks 1 and 2) or rowing on a Concept II rowing ergometer (weeks 3 and 4) at 70% peak \( \dot{V}O_2 \). Afternoon workouts on days 1, 3, and 5 of each week began with rowing for 15 min at 70% peak \( \dot{V}O_2 \); this was followed by three 2,500-m rowing time trials separated by 8-min recovery periods at a self-selected intensity. Power output during the time trials was used as an indicator of maximal performance. Afternoon workouts on days 2, 4, and 6 of each week began with 10 min of rowing ergometry at 70% peak \( \dot{V}O_2 \); this was followed by three sets of work-recovery intervals. The work intervals were at 90% peak \( \dot{V}O_2 \) and were performed in three sets that were separated by 5 min of recovery. The sets were 2 × 10, 3 × 5, and 4 × 3 min; within-set recovery intervals were 3, 2, and 1 min, respectively. All recovery intervals, between and within sets, were at a self-selected intensity. Peak \( \dot{V}O_2 \) was redetermined on days 7, 14, and 21, and training loads were readjusted as necessary for the following week. In addition, on days 14 and 21, after the peak \( \dot{V}O_2 \) test, rowing was performed continuously for 10, 15, and 10 min at 70, 80, and 70% peak \( \dot{V}O_2 \), respectively.

**Peak \( \dot{V}O_2 \) testing.** Peak \( \dot{V}O_2 \) was determined on the rowing ergometer before commencement of training. An incremental continuous protocol was used starting at ~50% peak \( \dot{V}O_2 \) followed by a 40-W increment and then 20-W increments. \( \dot{V}O_2 \) was determined with an automated open-circuit system that calculated \( \dot{V}O_2 \) every 30 s. The greatest measured \( \dot{V}O_2 \) was considered to be the subject's peak \( \dot{V}O_2 \).

**Blood sampling.** Blood samples were collected 3 days/wk in the fasted state before the morning workout; a final blood sample was taken 3 days after the last training session (day 29). Fifteen milliliters of blood were collected without anticoagulant, and 10 ml were collected with heparin sodium as anticoagulant. To determine hematocrit, three heparinized microcapillary tubes were filled with blood from the tube without anticoagulant; the remainder of this blood was allowed to clot on ice for 1 h. Tubes containing blood collected with heparin sodium were inverted several times and immediately immersed in an ice water bath for prompt cooling. Serum and plasma were separated by centrifugation (15 min at 1,000 g), aliquoted, and then stored at ~80°C until analysis.

**Muscle biopsies.** Muscle samples from the vastus lateralis were obtained on days 1, 10, 19, and 26, immediately before the afternoon time trials. Biopsies were taken from alternate legs on alternate weeks. Samples were quick-frozen in liquid nitrogen and stored at ~80°C until analysis.

**Analysis of blood and muscle.** Muscle damage and/or membrane permeability was assessed by measuring the serum activities of CK and a lysosomal enzyme, \( \beta \)-glucuronidase (\( \beta \)-Gluc). Malondialdehyde (MDA) is a degradation product of lipid peroxides of fatty acids that contain three or more double bonds and is most often measured using a thiobarbituric acid test (27). The results are expressed as MDA equivalents or as thiobarbituric acid-reactive substances (TBARS).

Serum CK activity was determined using a kit (47-UV, Sigma Diagnostics, St. Louis, MO). Triplicate spectrophotometric determinations of each sample had a mean coefficient of variation of 3.7%. Serum \( \beta \)-Gluc activity was assayed spectrophotometrically using a Sigma Diagnostics kit (Procedure No. 325). Intra- and interassay coefficients of variation were 3.6 and 4.4%, respectively.

Plasma TBARS (PTBARs) were assayed according to Yagi (37). Plasma (100 ml) was added to 4.0 ml of 0.083 M H\(_2\)SO\(_4\) in a screw-cap tube, and 500 ml of 10% phosphotungstic acid were then added to precipitate the proteins and lipids. After 5 min at room temperature, the mixture was centrifuged (1,700 g) for 5 min at 4°C. After the supernatant was discarded, the pellet was washed in 2 ml of 0.083 M H\(_2\)SO\(_4\) and 300 ml of 10% phosphotungstic acid, vortexed, and then centrifuged as above. The supernatant was discarded, and the pellet was resuspended in 1.0 ml of a solution of glacial acetic acid and 0.67% thiobarbituric acid (1:1 vol/vol, prepared just before use). The tubes were tightly capped with Teflon-lined screw caps, incubated for 1 h at 100°C in a dry heat block, and cooled in a water bath to room temperature. Next, 4 ml of n-butanol were added. The tubes were shaken for 2 min on a Vortex Genie mechanical shaker and then centrifuged as
above. Fluorescence of the supernatant was determined on a fluorometer (Perkin-Elmer LS-5B) with excitation-emission wavelengths set at 531-547 nm, respectively, and slits set at 3 nm. Full-scale fluorescence was set to 100 units by use of the highest standard. A standard curve over the range 20-300 nmol/ml was generated using 1.1.3.3-tetraethoxypropane, which quantitatively yields MDA under the conditions of the assay (15). All samples were assayed in triplicate on a single day with a single batch of reagents. A plasma pool assayed three times during h resulted in a coefficient of variation of 9.8%.

Assay for muscle TBARS (MTBARS) was contingent on the availability of a sufficient quantity of muscle after determination of glycogen from the biopsy sample. MTBARS were assayed using the procedure of Ohkawa et al. (26). Muscle (5-15 mg) was homogenized (5% wt/vol) by hand, glass-on-glass, in 1.15% KCl. To 25-75 µl of homogenate were added 200 µl of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid (pH 3.5, adjusted with NaOH), and 1.5 ml of 0.8% thiorbituric acid. Water was added to produce a final volume of 4.0 ml. After a 1-h incubation at 100°C, the reaction tubes were cooled to room temperature in a water bath, and 2.0 ml of n-butanol-pyridine (15:1, vol/vol) were added. Subsequent steps were as outlined for PTBARS, except the excitation-emission wavelength pair was 531-563 nm. Intra-assay coefficient of variation for triplicate determinations was 2.3%. Results for MTBARS were normalized to protein content and expressed as nanomoles per milligram of protein. Protein concentration of the homogenate was determined using the dye-binding assay described by Bradford (10), with bovine serum albumin as standard. The mean coefficient of variation for duplicate analyses was <1%.

Statistical analysis. The data were analyzed with a two-way analysis of variance with repeated measures. The alpha level of 0.05 was used for all statistical tests. When appropriate, Tukey's post hoc test was used to locate differences between means. Correlational analysis was used to characterize linear relationships between the serum and plasma markers of oxidant stress and muscle damage.

RESULTS

Power output and muscle glycogen. The HI group exhibited significantly greater mean power outputs over the entire 4 wk than did the MOD group. Both groups improved their power outputs over the course of training, but the HI group improved 10.7 ± 1.0% (SE), whereas the MOD group improved 1.6 ± 1.6%. On day 26, the final training day, the HI group produced 7.8% more power than did the MOD group (P < 0.05). The MOD group maintained an average muscle glycogen concentration of 119 mmol/kg throughout the 4 wk of training, but muscle glycogen concentration in the HI group increased 65% from 94 ± 7 mmol/kg on day 1 to 155 ± 10 mmol/kg (P < 0.05) on day 26, at which time it was also greater than that for the MOD group (P < 0.05).

Training intensity. The mean percentage of maximum heart rate obtained by all rowers during the three 2,500-

FIG. 1. Percent change in serum creatine kinase activity in men and women rowers who underwent 4 wk of twice-daily high-intensity rowing training while consuming each day isocaloric diets containing 5 (MOD) or 10 (HI) g carbohydrate/kg body wt. Values are means ± SE (n = 22). There was a main effect of time (P < 0.05), but post hoc comparisons among means were nonsignificant.

in time trials ranged from 91.3 ± 0.6 to 98.7 ± 0.4%. The rowers were able to maintain the desired training intensity (70-90% peak VO2) throughout the 4-wk training period. Similarly, heart rates for the other training sessions were within the desired range for the prescribed intensity (data not shown). That training stressed the muscle glycogen supply was demonstrated by pilot data on one subject showing that a time-trial training session required the degradation of 114 mmol of glycogen per kilogram of wet muscle weight. On the basis of the duration and intensity of training, a similar magnitude of glycogen degradation presumably occurred during the other afternoon sessions.

Serum CK activity. Serum CK activities were consistently lower for the women than for the men, with an overall average difference of 55 ± 12%. Therefore, to depict the response of CK activity to training, we have reported the percent change from day 1 (Fig. 1). It is obvious from Fig. 1 that the pattern of changes in serum CK activity in response to training was essentially identical for men and women and for both diet groups. Although there was a significant main effect of time (i.e., training) for changes in CK activity, presumably because of the relatively greater mean changes during the 1st wk of training, no significant post hoc pairwise mean differences among days were detected.

Plasma β-Gluc activity. Because subjects of both sexes and both dietary treatments had similar β-Gluc responses to training and because of the great variability in the data, only pooled results are shown in Fig. 2. A signifi-
FIG. 2. Serum β-glucuronidase activity in pooled data for men and women rowers who underwent 4 wk of twice-daily high-intensity rowing training while consuming each day isocaloric diets containing 5 or 10 g carbohydrate/kg body wt. Values are means ± SE (n = 22). There was a main effect of time (P < 0.05).

cant effect of time (i.e., training) without post hoc differences was detected; the average β-Gluc activity was ~10% greater on days 6–16 than during earlier and later stages of training.

PTBARS. The PTBARS analysis detected significant main effects of time (training) and a significant diet-by-sex interaction without significant post hoc differences (Fig. 3). In general, most of the collapsed means for gender and treatment were somewhat greater on days 1 and 30 and somewhat lower on days 9 and 13. Also the men who consumed a high-carbohydrate diet had slightly greater PTBARS values than the other subgroups for most of the final 3 wk of training.

MTBARS. Because all the tissue in the muscle biopsy samples was sometimes required for the muscle glycogen assays, 10 tissue samples for men and 12 for women were unavailable for MTBARS assay; furthermore, these unavailable samples were not evenly distributed between diet groups. Therefore, because the two-way analysis of variance procedure requires complete data, statistical analysis was not performed on MTBARS data, and data for the two diets were pooled to display the available results by gender in Fig. 4. The female rowers had lower MTBARS values than did the male rowers, but there was no apparent systematic effect of the rowing training on MTBARS and no apparent gender-related effect on MTBARS response to training. No significant linear relationship among the blood-borne markers of oxidant stress and muscle damage was detected by correlational analysis.

DISCUSSION

Appearance in the blood of muscle-derived enzymes indicates the presence of muscle damage or increased permeability of muscle membranes (5, 18). The changes in serum CK activity observed in this study are unremarkable throughout the 4-wk training period, perhaps because eccentric contractions are not heavily utilized in cycling and rowing activities. A minor increase in CK activity in the 1st wk of the study was probably caused by the sudden imposition of the twice-daily training. However, if a chronic oxidant stress had been provoked by the training, a chronically elevated CK activity in serum would have been expected. Such an elevation was not observed. Similarly, there were no sustained increases in the PTBARS and β-Gluc indicators of oxidant stress. The times for blood sampling were always ~12 h after a time-trial workout (except on days 1 and 29). The early-morning postprandial preworkout timing of blood draws was chosen over an immediate posttraining sampling because of the desire to focus on chronic oxidant stress responses and thus to eliminate an acute response to a meal or to a training session. After exercise, TBARS and β-Gluc remain elevated for >12 h (24), and the peak CK response is delayed 12–48 h after muscle-damaging exer-
exercise (24). Maughan et al. (24) demonstrated that although TBARS peaked 6 h after a downhill treadmill run, the TBARS concentration was still elevated 24 and 48 h after exercise. Interestingly, Maughan et al. also observed that subjects classified as high CK responders had greater preexercise TBARS concentrations than did low CK responders. Jackson et al. (17) were able to correlate CK efflux with an increased free radical signal in muscle after extensive contractile activity in rat hindlimbs. Thus the lack of sustained PTBARS, CK, and β-gluc responses could be considered confirmation that a chronic oxidant stress did not occur in rowers undertaking intense twice-daily aerobic training. Finally, although absolute values for serum CK activity were lower for the women than for the men in this experiment, there were no gender-associated differences in the pattern of CK response to rowing training. Thus, although women (8) and female rats (3) exhibited reduced acute CK responses to exercise relative to their male counterparts, we found no evidence of this phenomenon in the present chronic training experiment.

We hypothesized that the diets and training regimen would result in different glycogen concentrations between the MOD and HI groups and that a greater glycogen concentration in the HI group would reduce oxidant stress and increase rowing power output. The pretraining glycogen values for the HI group were greater than those for the MOD group, and the time-trial power outputs of the HI rowers were 7.3% greater than those for the MOD rowers at day 26. Each group improved power output over the course of training, with the HI group improving somewhat more than the MOD group. This ability to train intensely and to improve or maintain power output in both groups of rowers also argues against the occurrence of oxidant stress. However, the failure to detect the products of oxidant stress may only reflect an enhanced capacity for degrading or metabolizing oxidant stress products (32). In fact, training-induced adaptations of enzymatic antioxidant defense systems have been documented (31). The present study did not evaluate the antioxidant defense systems.

The significant PTBARS diet-by-sex interaction is not readily explained. The male HI group had greater PTBARS concentrations than the other rowers after the 4th day of training. It is possible that a diet effect occurred independently of a training effect. To gain further insight into this possibility, we repeated the MOD and HI diet protocols for 7 days on a runner training for 60 min/day at 75% peak VO2. The diet cross-over trials were conducted at 1-mo intervals. The runners' PTBARS were not higher while on the HI diet. Finally, hematocrit differences cannot explain the PTBARS difference, because hematocrit percentage did not differ between the male diet groups.

The MTBARS results only suggest that 1) there is no apparent training-induced change in MTBARS and 2) the apparently lower MTBARS data for the women rowers than for the men mimics the gender-related differences in the CK data. The women trained at the same relative intensity as the men, but in comparison to the men, they had lower MTBARS and serum CK values, perhaps because of a stabilizing effect of estrogens on cell membranes (25, 36).

In summary, men and women rowers were able to train at a high intensity for 4 wk without demonstrating significant indexes of oxidant stress. Although the absolute values of serum CK activity and MTBARS were lower in the women than in the men, no gender-related differences in the response of these and other variables to training were observed. HI rowers exhibited greater muscle glycogen stores and greater power outputs than MOD rowers, but the diets did not influence indexes of oxidant stress.

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