

## Plasma Protein Carbonyl Response to Increasing Exercise Duration in Aerobically Trained Men and Women

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### **Abstract:**

The purpose of this study was to determine the effect of aerobic exercise duration on plasma protein carbonyl concentrations, a marker of protein oxidation, in aerobically trained men and women. Eight men (age:  $27 \pm 4$  years,  $V \cdot O_{2\text{peak}}$ :  $4.09 \pm 0.26 \text{ L} \cdot \text{min}^{-1}$ ; mean  $\pm$  SD) and 7 women (age:  $27 \pm 6$  years,  $V \cdot O_{2\text{peak}}$ :  $2.33 \pm 0.24 \text{ L} \cdot \text{min}^{-1}$ ) exercised on an electrically-braked cycle ergometer at 70 %  $V \cdot O_{2\text{peak}}$  for 30, 60 or 120 minutes on three separate days. Plasma samples collected before and immediately, 30- and 60-minutes post-exercise were analyzed for protein carbonyls. Mean oxygen uptake was greater for men in all conditions ( $2.75 \pm 0.03 \text{ L} \cdot \text{min}^{-1}$ ;  $38 \pm 0.43 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) compared to women ( $1.57 \pm 0.03 \text{ L} \cdot \text{min}^{-1}$ ;  $24.1 \pm 0.47 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ). Total work performed during the exercise sessions was also greater for men than for women during the 30 ( $368 \pm 11$  versus  $223 \pm 7$  kJ), 60 ( $697 \pm 17$  versus  $423 \pm 18$  kJ), and 120-minute conditions ( $1173 \pm 44$  versus  $726 \pm 28$  kJ) (Mean  $\pm$  SEM). Although these comparisons were significant ( $p < 0.0001$ ), sex differences in total work performed and mean  $V \cdot O_2$  did not result in sex differences in protein carbonyls. However, a condition by time interaction was observed with greater post-exercise values following the 120-minute condition compared to both the 30- and 60-minute conditions. Protein carbonyl concentration was greatest immediately post-exercise for both men and women and generally declined in a linear trend through one hour of recovery. These data suggest that protein carbonyl concentration is elevated by cycling exercise performed at 70 %  $V \cdot O_{2\text{peak}}$ , is greater following longer duration rides, begins to recover within one hour following exercise, and is not different between men and women.

### **Key words**

Oxidative stress - cycling - reactive oxygen species

### **Introduction**

Proteins possess multiple physiologic functions within biological systems; their exact conformation and structure are tightly connected to their function and activity [[4]]. The generation of reactive oxygen and nitrogen species (RONS) such as singlet oxygen ( $\text{O}^1$ ), superoxide radical ( $\text{O}_2^{\cdot-}$ ), hydroxyl radical ( $\text{OH}^{\cdot}$ ), and peroxynitrite ( $\text{ONO}_2^{\cdot-}$ ) occurs as a consequence of normal cellular metabolism, and appears to be increased under conditions of both physiological and psychological stress [[8]]. Aerobic exercise, one such physiological stressor, can lead to increased oxidative cellular damage, likely due to enhanced production of RONS [[15]]. The increase in RONS with aerobic exercise has been attributed largely to disruption of

the electron transport system. This leads to increased leakage of superoxide radicals and other phenomena, such as catecholamine auto-oxidation and prostanoid metabolism [[6]]. In addition, skeletal muscle injury and the associated physiological changes that often accompany such injury (e.g., inflammation, proteolysis, loss of calcium homeostasis) may further increase RONS production and subsequent macromolecule oxidation [[3]]. When RONS production exceeds the body's protective capabilities by way of antioxidant defense mechanisms, a condition of oxidative stress may result, leading to modifications of macromolecules including lipids, nucleic acids, and proteins [[15]]. RONS can react directly with proteins or they can react with molecules such as lipids or sugars, generating secondary products, which may then react with the proteins. When exposed to prooxidant species in the presence of transition metal ions, either the peptide bond or the protein side chain may be targeted, as all amino acids are vulnerable to metal-catalyzed oxidation [[15]]. Oxidation reactions may involve specific modifications (e.g., conversion of phenylalanine residues to *o*-tyrosine and of tyrosine to dityrosine) or more global modifications as in the conversion to carbonyl derivatives, leading to loss of catalytic or structural function in the affected proteins and making them highly susceptible to proteolytic degradation [[10]]. Further, the susceptibility to oxidation may be protein specific, such that certain proteins are more likely to undergo oxidation than others. In relation to disease states, oxidized proteins appear to accumulate in cells and in certain pathological states can account for over 50 % of the total cellular protein content [[8]]. In this case, the level of dysfunctional proteins is so high that they may actually be a major contributing cause of the disease rather than merely a consequence. It should be noted, however, that despite the potential negative consequences of RONS, a certain level of production appears necessary for the purpose of cell signaling and normal physiological processes [[17]].

The magnitude of the oxidative modifications to macromolecules appears related to the intensity of the aerobic exercise session, a finding well supported by the biomarker malondialdehyde, a measure of lipid peroxidation [[9], [12]]. In addition, exercise duration may be an important factor related to RONS production, as DNA oxidation has been reported to be increased in response to prolonged exercise duration in both men and women [[2], [16]]. Little is known regarding the impact of exercise duration pertaining to protein oxidation. To our knowledge, only Goldfarb et al. [[5]] has compared protein oxidation resulting from aerobic exercise between men and women. In this study, protein carbonyls were noted to be elevated following 30 minutes of cycling at both 60 % and 80 %  $V\cdot O_{2max}$  in both men and women, with no differences noted between sexes. However, this study did not compare protein carbonyl levels following different exercise durations. Therefore, the purpose of the present study was two-fold: first, to compare the extent of protein oxidation during the one hour following aerobic exercise of three different durations and, second, to compare the protein oxidation response between men and women. We hypothesized that protein carbonyls would be greater following the longer duration bouts and that the response would be similar for both men and women, as previously demonstrated for DNA oxidation.

## **Methods**

### *Subjects*

Eight men and seven women (aged 18 - 35 years) volunteered to participate as subjects. Men were regularly performing aerobic exercise an average of  $10.4 \pm 1.6$  (mean  $\pm$  SD) hours per week and women performed aerobic exercise an average of  $4.5 \pm 2$  hours per week. While no subject

was competitive at the state or national level, several subjects were locally competitive in cycling and running events, with some being former or current collegiate athletes. A medical history and physical activity questionnaire were completed by all subjects in order to determine eligibility. All subjects were free of orthopedic and metabolic conditions that could have affected the variables of measurement. Furthermore, no subject was a smoker or used oral contraception or anti-inflammatory drugs. The University Institutional Review Board approved all experimental procedures and subjects signed an informed consent form prior to enrollment in the study.

### *Experimental procedures*

At least one week before the first submaximal exercise session, measurements of resting heart rate, height, weight and estimation of percent body fat by seven skinfold sites [[7]] were performed. Subjects then completed a maximal graded exercise test on a Lode Excalibur electronically braked cycle ergometer (Lode BV, Medical Technology, Groningen, The Netherlands) for determination of maximal oxygen consumption ( $\dot{V}\cdot\text{O}_{2\text{peak}}$ ) using a SensorMedics  $\dot{V}\cdot\text{max}$  229 metabolic cart (SensorMedics, Yorba Linda, CA, USA). Following a brief warm-up period, subjects pedaled between 70 - 100 revolutions per minute (RPM), starting at 100 Watts for men and 50 Watts for women. Workrate increased 50 Watts every two minutes for men and 25 Watts every two minutes for women until volitional fatigue. The highest mean one-minute  $\dot{V}\cdot\text{O}_2$  value obtained during testing constituted  $\dot{V}\cdot\text{O}_{2\text{peak}}$  and was used to calculate the submaximal workrate.

Submaximal exercise testing was completed in the morning (06:00 - 08:00h) on three separate days after an eight-hour fast. All women were tested during the early follicular phase of the menstrual cycle (days 2 - 8) to minimize variance in estrogen levels. The three exercise conditions consisted of cycling at 70 %  $\dot{V}\cdot\text{O}_{2\text{peak}}$  for 30, 60 or 120 minutes (randomized order).  $\dot{V}\cdot\text{O}_2$  was assessed every fifteen minutes and, if necessary, workrate was adjusted in order to maintain 70 %  $\dot{V}\cdot\text{O}_{2\text{peak}}$ . Total work was calculated using the following equation: (average Watts · total minutes of cycling)/16.6 = total work (kJ).

### *Blood collection and analysis*

Blood samples for analysis of plasma protein and protein carbonyls were collected from an antecubital vein via an indwelling catheter into vacutainer tubes containing EDTA solution. Samples were taken pre-exercise and immediately (0-), 30-, and 60-minutes post-exercise. Blood was immediately placed on ice and centrifuged at 3000 rpm for 15 min at 4 °C in a Beckman (J2-21) centrifuge (Beckman Coulter, Fullerton, CA, USA). Plasma was stored at - 80 °C until analyzed. All samples were analyzed in duplicate at first thaw.

Plasma protein was determined by the method of Lowry et al. [[13]], comparing values to known standards. Following adjustment to 4 mg · mL<sup>-1</sup> protein using phosphate buffer, protein carbonyls were determined from plasma using the 2,4-dinitrophenylhydrazine (DNPH) spectrophotometric method described by Levine et al. [[11]]. Briefly, samples containing either 2 N HCL or DNPH were passed through columns containing Sephadex G 10 and rinsed with 2 N HCl. The effluent was collected, mixed with guanidine HCL, and the absorbance determined at 360 nm on a Shimadzu UV-1601 spectrophotometer (Shimadzu, Columbia, MD, USA). For all samples, the change in absorbance with and without DNPH was calculated. Values are expressed as molar quantities using the extinction coefficient 22 000 M<sup>-1</sup> · cm<sup>-1</sup>, in nM · mg · protein<sup>-1</sup>.

### Statistical analyses

The data for protein carbonyls were analyzed using a 2 (sex) by 3 (exercise condition) by 4 (time) repeated measures ANOVA. Significant interactions and main effects were further analyzed using Tukey's *post hoc* tests. Submaximal exercise data ( $V\cdot O_2$ , kcal, work, and HR) were analyzed using a 2 by 3 ANOVA. In an attempt to determine the contribution of total work and total  $V\cdot O_2$  on protein carbonyl levels, a bivariate fit of both the total work and the total  $V\cdot O_2$  against the peak protein carbonyl response for each subject, and each sex, was performed. Lines of best fit and corresponding 95 % confidence interval bands were constructed around the lines for analysis of statistical differences between slopes for men and women. Subject characteristics were compared between men and women using a one-way ANOVA. All analyses were performed using JMP statistical software version 4.0 (SAS Institute, Cary, NC, USA). Statistical significance was set at  $p < 0.05$ . The data are presented as mean  $\pm$  SEM, except for subject characteristics (Table [1]), which are presented as mean  $\pm$  SD.

Table 1 Characteristics of eight men and seven women participants

Variable	Men (mean $\pm$ SD)	Women (mean $\pm$ SD)	P value
Age (yrs)	27 $\pm$ 4	27 $\pm$ 6	0.989
Height (cm)	180 $\pm$ 6	170 $\pm$ 5	0.004
Weight (kg)	71.9 $\pm$ 9.0	64.8 $\pm$ 6.9	0.115
Body mass index (kg $\cdot$ m <sup>-2</sup> )	22.3 $\pm$ 2.1	22.5 $\pm$ 3.4	0.872
Percent body fat	11.3 $\pm$ 2.9	18.3 $\pm$ 5.5	0.008
Resting heart rate (bpm)	63 $\pm$ 6	59 $\pm$ 7	0.285
$V\cdot O_{2peak}$ (L $\cdot$ min <sup>-1</sup> )	4.09 $\pm$ 0.26	2.33 $\pm$ 0.24	< 0.0001
$V\cdot O_{2peak}$ (ml $\cdot$ kg <sup>-1</sup> $\cdot$ min <sup>-1</sup> )	57.4 $\pm$ 5.3	36.5 $\pm$ 5.8	< 0.0001
Aerobic exercise (h $\cdot$ wk <sup>-1</sup> )	10.4 $\pm$ 1.6	4.5 $\pm$ 2	< 0.0001

### Results

All subjects completed three testing conditions. However, two of the men failed to complete the 120-minute ride, but cycled until exhaustion (92  $\pm$  2 min). These two subjects remained in the analysis, as their protein carbonyl response was similar to all other subjects following the 120-minute ride. Both men and women cycled within 1 ml  $\cdot$  kg<sup>-1</sup>  $\cdot$  min<sup>-1</sup> of their 70 %  $V\cdot O_{2peak}$  values during all exercise conditions. Mean oxygen uptake was greater for men in all conditions (2.75  $\pm$  0.03 L  $\cdot$  min<sup>-1</sup>; 38  $\pm$  0.43 ml  $\cdot$  kg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>) compared to women (1.57  $\pm$  0.03 L  $\cdot$  min<sup>-1</sup>; 24.1  $\pm$  0.47 ml  $\cdot$  kg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>). The total amount of work performed during the exercise sessions was also greater for men than for women during the 30- (368  $\pm$  11 versus 223  $\pm$  7 kJ), 60- (697  $\pm$  17 versus 423  $\pm$  18 kJ), and 120-minute conditions (1173  $\pm$  44 versus 726  $\pm$  28 kJ). All of the above comparisons were significant at the  $p < 0.0001$  levels.

### Protein carbonyls

There was no 3-way interaction for protein carbonyls as noted in Fig. [1] ( $p = 0.8673$ ). Additionally, there was no sex by time ( $p = 0.6051$ ) or sex by condition ( $p = 0.6707$ ) interactions. A condition by time interaction was noted for protein carbonyls as shown in Fig. [2]

( $p = 0.0028$ ), with values greater at all times post-exercise compared to pre-exercise following the 120-minute condition, and greater immediately and 30-minute post-exercise compared to pre-exercise following both the 30- and 60-minute conditions. Additionally, values were greater at all times post-exercise following the 120-minute condition compared to the 30 and 60-minute conditions. No differences existed in protein carbonyls between the 30- and 60-minute conditions at any time. For all exercise conditions, values reached a peak immediately post-exercise, increasing 161 %, 197 %, and 245 % following the 30-, 60-, and 120-minute rides, respectively.

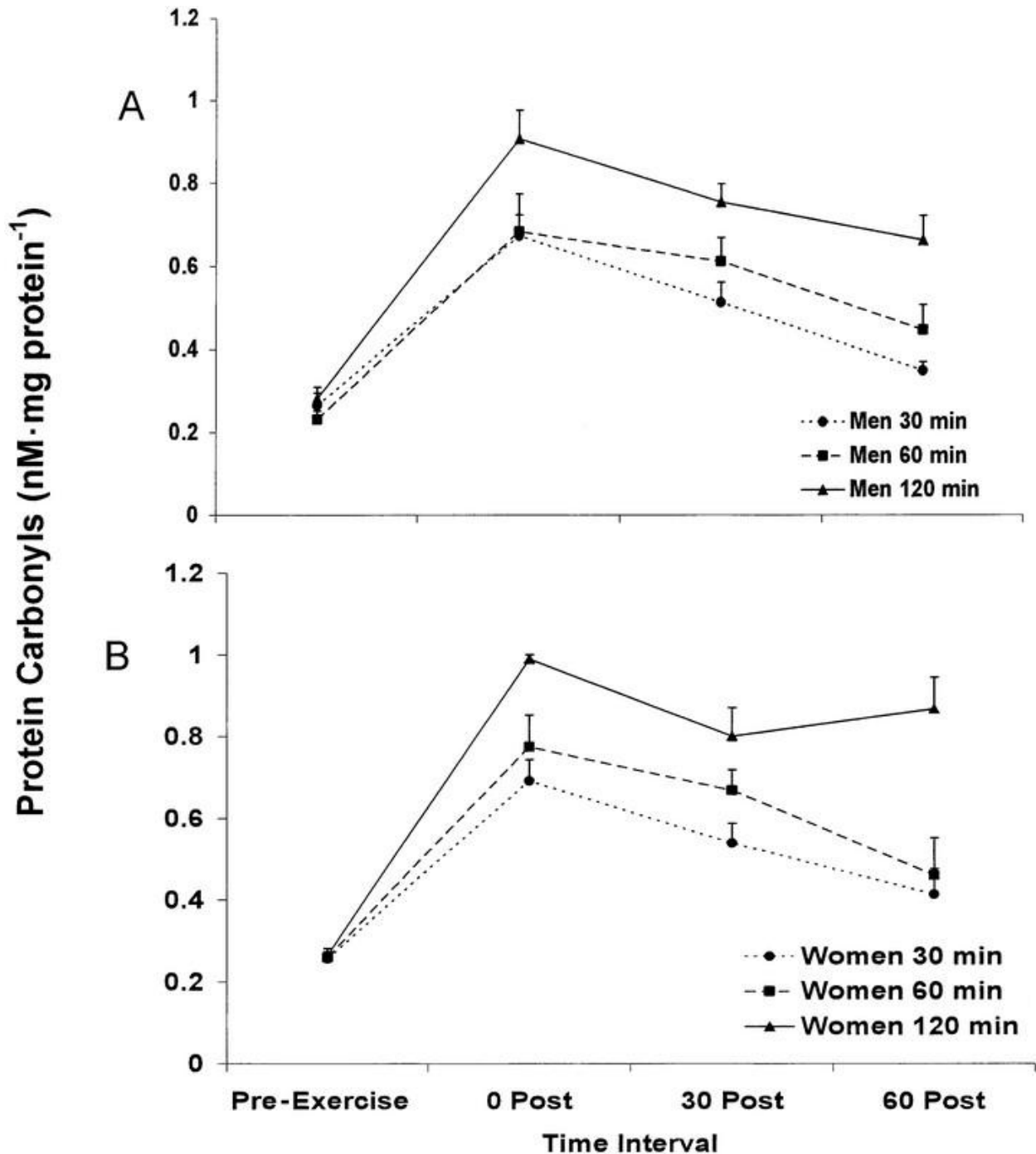


Fig. 1 **A** and **B** Plasma protein carbonyls of men (**A**) and women (**B**) before and following 30, 60, and 120 minutes of cycling at 70 %  $V\cdot O_{2peak}$  (mean  $\pm$  SEM). No differences were present between men and women within any trial at any time point ( $p > 0.05$ ).

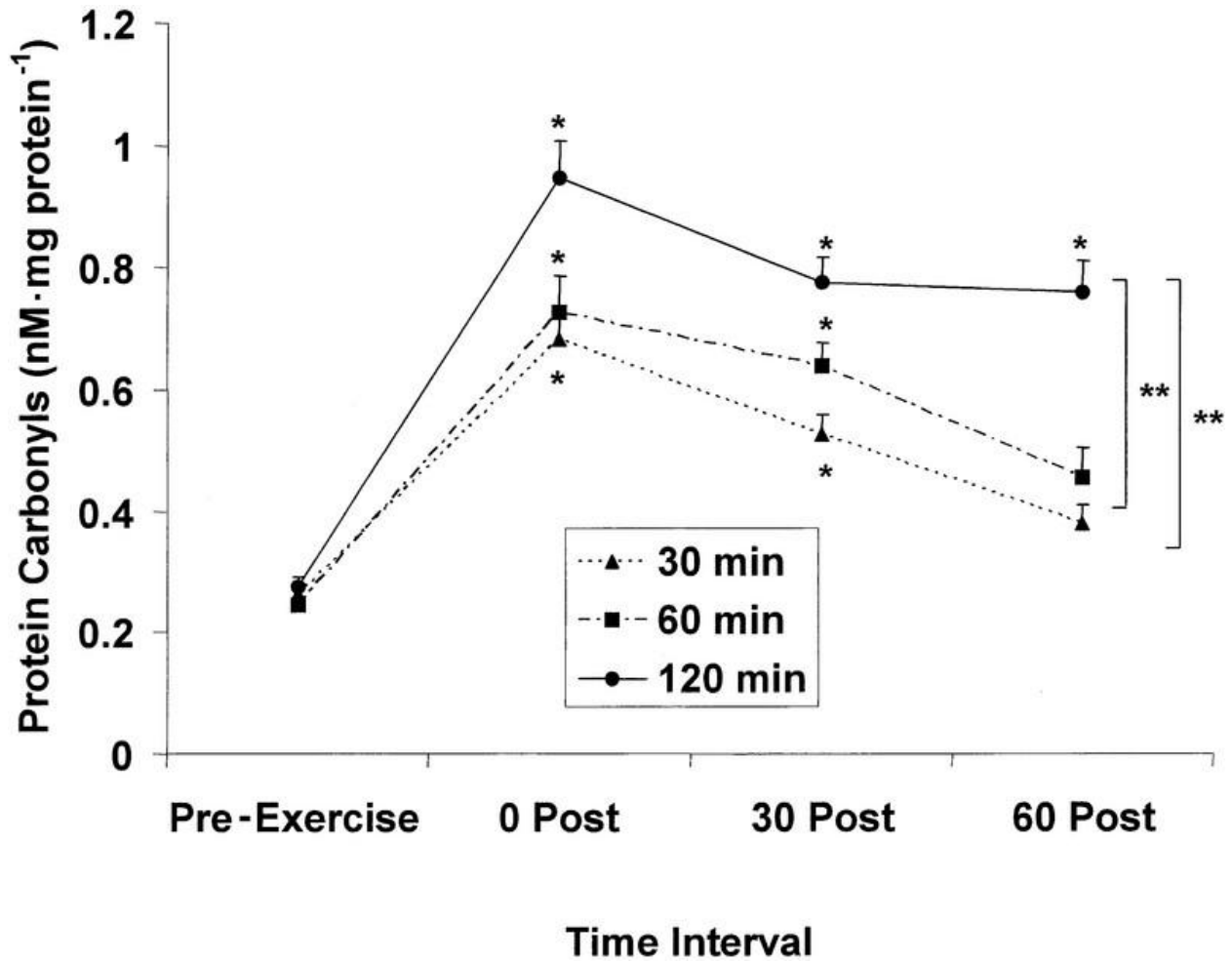


Fig. 2 Plasma protein carbonyls collapsed over eight men and seven women before and following 30, 60, and 120 minutes of cycling at 70 %  $V\cdot O_{2peak}$  (mean  $\pm$  SEM). \* Protein carbonyl concentration greater than pre-exercise ( $p < 0.05$ ). \*\* Protein carbonyl concentration greater at all times post-exercise for the 120-min condition compared to the 30 and 60 min conditions ( $p < 0.05$ ); No difference between the 30- and 60-min conditions at any time post-exercise ( $p > 0.05$ ).

No differences existed between men or women with regards to the peak protein carbonyl levels, as a function of either total work or total  $V\cdot O_2$ . However, while not statistically different, it appeared that the total amount of work performed explained a greater amount of the peak protein carbonyl levels in women ( $R^2 = 0.32$ ) than in men ( $R^2 = 0.20$ ). This was also true for total  $V\cdot O_2$ , yielding  $R^2$ -values of 0.25 and 0.12 for women and men, respectively (Fig. [3], [4]).

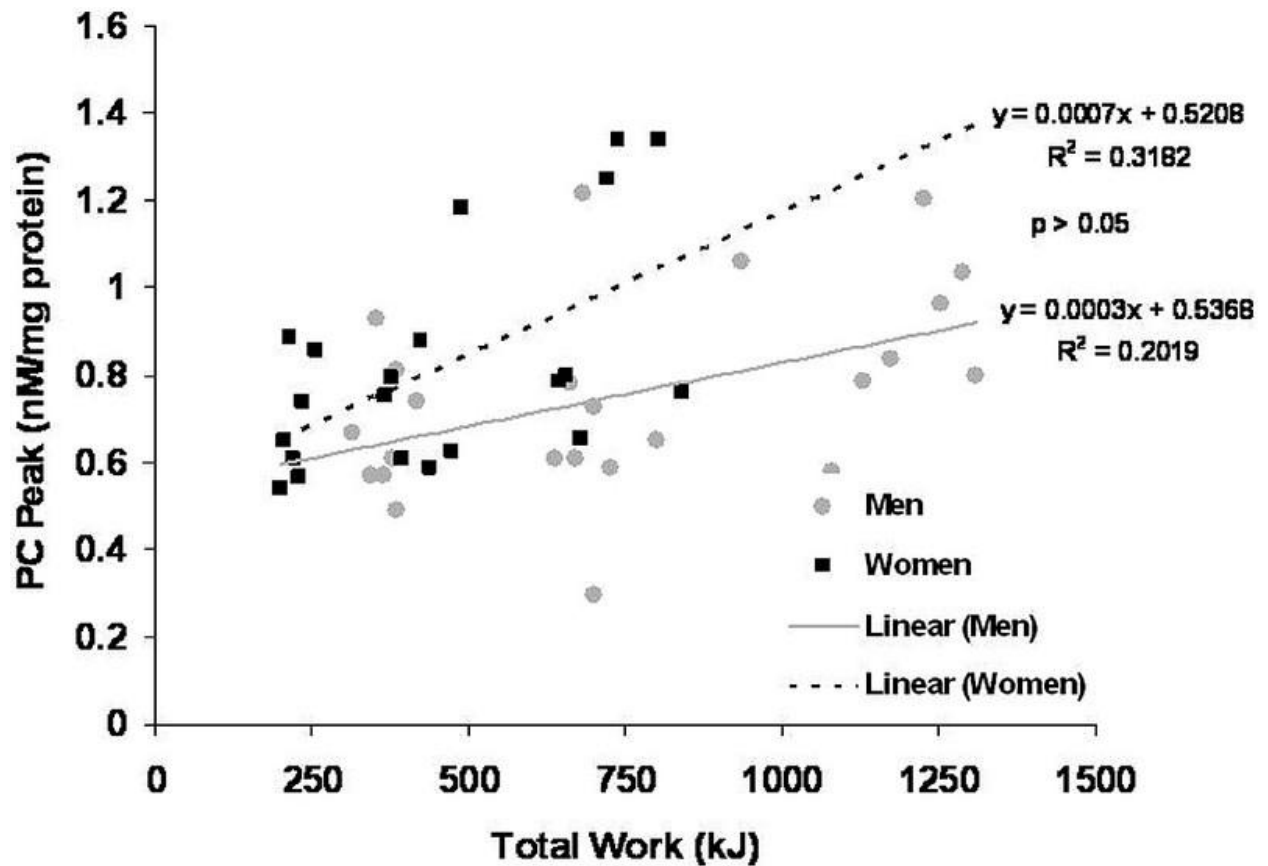


Fig. 3 Peak protein carbonyl values as a function of total work for men and women. Lines of best fit are included. No statistically significant difference was noted between men and women ( $p > 0.05$ ).

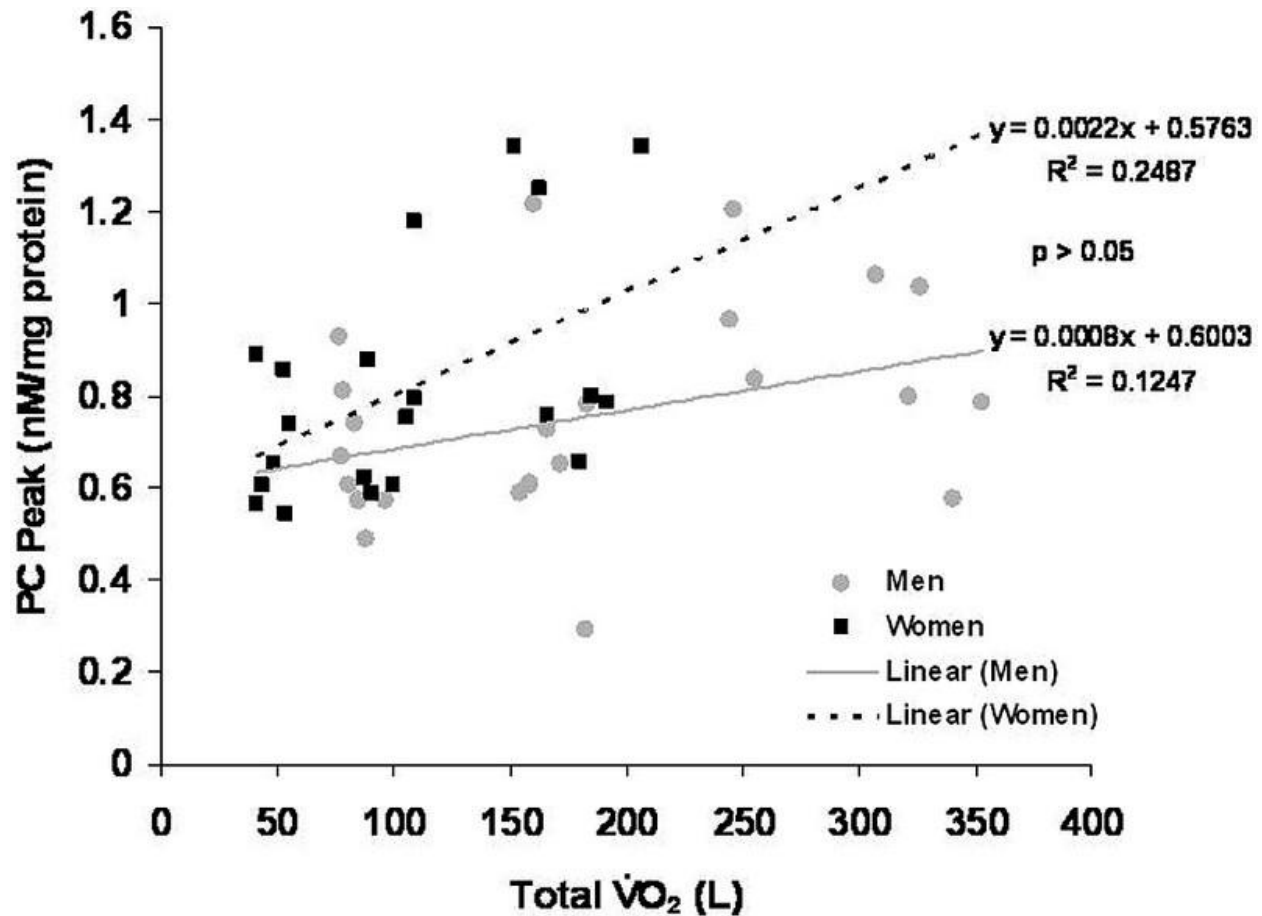


Fig. 4 Peak protein carbonyl values as a function of total  $\dot{V}O_2$  for men and women. Lines of best fit are included. No statistically significant difference was noted between men and women ( $p > 0.05$ ).

### Discussion

The main findings of this investigation are: 1) Protein oxidation, assessed through plasma protein carbonyl concentration, is elevated to a greater extent following longer versus shorter duration cycling performed at 70 %  $\dot{V}O_{2peak}$ , 2) the rise in protein carbonyls is transient and declines during the first hour after exercise, and 3) there are no sex differences in the protein carbonyl response to exercise.

To our knowledge, this was the first study to compare protein oxidation between men and women following different exercise durations. Only one study presented in abstract form has compared protein oxidation following aerobic exercise with regards to sex [[5]]. In the study by Goldfarb et al [[5]], subjects cycled for 30 minutes at both 60 and 80 %  $\dot{V}O_{2max}$ . While protein carbonyls were higher following the 80 % ( $1.20 \text{ nM} \cdot \text{mg} \cdot \text{protein}^{-1}$ ) versus the 60 %  $\dot{V}O_{2max}$  condition ( $0.60 \text{ nM} \cdot \text{mg} \cdot \text{protein}^{-1}$ ), there was no sex difference in values immediately post-exercise. The mean protein carbonyl values for subjects in the present investigation were far lower following 30 minutes of cycling ( $0.682 \text{ nM} \cdot \text{mg} \cdot \text{protein}^{-1}$ ) than those reported by Goldfarb et al. [[5]] when untrained subjects cycled at 80 %  $\dot{V}O_{2max}$ . It is possible that our slightly lower exercise intensity could have been a factor in mediating the lower protein carbonyl



concentrations. Additionally, subjects' training status could have impacted blood protein oxidation. This effect could have been mediated by an upregulation in antioxidant enzyme activity as previously reported following chronic aerobic exercise training [[14]]. Therefore, data from the present investigation may apply specifically to those who are aerobically trained, while sedentary individuals may experience an exaggerated increase in protein carbonyls following acute exercise. Conversely, men and women in the present study had similar elevations in protein carbonyl concentrations following exercise, despite a significantly greater training volume practiced by the men. However, *both* the men and women were highly active. It is possible that the training-induced attenuation of protein oxidation in response to acute exercise plateaus at higher training volumes.

In the present study, protein carbonyls were elevated post-exercise regardless of duration. These results are similar to those of Alessio et al. [[1]], who reported an increase in protein carbonyls immediately following a graded treadmill protocol, with values approaching baseline within one hour post-exercise. Protein carbonyl production was greatest following the 120-minute condition, where the total work completed and the oxygen consumption was highest. Since increased oxygen consumption is believed to be a major contributor to the generation of RONS [[6]], it is tempting to speculate that protein oxidation following aerobic exercise is directly related to the weight-relative amount of oxygen consumed. However, careful consideration of the extent of protein carbonyl formation between sexes appears to refute this hypothesis. That is, both men and women experienced a similar rise in protein carbonyls following the three different durations of exercise, despite the fact that men consumed much more oxygen than did women.

Additionally, the  $R^2$ -value associated with total oxygen consumption in relation to the peak protein carbonyl response was only 0.25 for women and 0.12 for men. Likewise, the contribution of total work performed, while slightly greater than total  $V \cdot O_2$ , was also relatively low (women:  $R^2 = 0.32$ ; men:  $R^2 = 0.20$ ). Therefore, rather than weight-relative oxygen uptake, we speculate that protein carbonyl formation may be more dependent on the maintenance of a relative percentage of  $V \cdot O_{2peak}$  over different exercise durations (i.e., an accumulation effect). It was noted that both men and women cycled at approximately 70 %  $V \cdot O_{2peak}$  for the 30-, 60-, and 120-minute periods. These similar *relative* workrates corresponded to similar degrees of protein oxidation observed as protein carbonyls in the plasma. While overall oxygen uptake and total work was indeed higher in men than in women (even when controlling for the amount of lean body mass), the protein carbonyl response was similar, lending support to our hypothesis that relative exercise intensity expressed as a percentage of  $V \cdot O_{2peak}$  and maintained for varying lengths of time may ultimately dictate the extent of protein oxidation following aerobic exercise.

However, considering the complexity of the human system, this factor alone cannot be expected to explain the protein carbonyl levels observed here. It is likely that multiple metabolic factors, including those not measured in the present study (e.g., muscle damage, proteolysis, prostanoid metabolism, inflammation), contributed to the protein carbonyl findings. Using an aerobic exercise stimulus, it is possible that the oxidized proteins observed might have been structural, contractile, or enzymatic in origin. Future investigations may choose to isolate these causative factors, as well as the type and location of the oxidized proteins in an attempt to better understand the role of each factor in promoting protein carbonyl formation.

While protein carbonyls were greater following the 120-minute condition compared to the 30- and 60-minute conditions, similar findings were noted between the 30- and 60-minute rides. It is possible that an exercise duration threshold is necessary for further production of protein carbonyls (i.e., > 60 minutes of continuous exercise), possibly through increased ATP production and subsequent RONS formation through the mitochondria electron transport chain. Protein carbonyls following the 120-minute condition remained elevated for a longer time course compared to the shorter duration conditions. In fact, because samples were not taken beyond 60-minutes post-exercise, it is unknown whether or not these elevations would have persisted. However, because values were generally declining at this time with the exception of a few women following the 120-minute condition, this possibility is unlikely. Additional research is needed to more fully elucidate the role of exercise duration on protein oxidation following aerobic work.

In conclusion, protein carbonyl concentration is transiently elevated following cycling exercise performed at 70 %  $\dot{V}\cdot O_{2peak}$ . Additionally, the response is greater following longer duration exercise, and is similar for both men and women. While chronic elevations in oxidized plasma proteins may prove a health hazard [[8]], it is unknown as to what, if any, health consequences are associated with the short-lived changes in protein carbonyls as observed in the present study. This is especially true in light of observations suggesting the necessity of homeostatic levels of RONS to regulate muscle tissue remodeling and cell signaling [[17]].

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