Generation of reactive oxygen species after exhaustive aerobic and isometric exercise

HELAINE M. ALESSIO, ANN E. HAGERMAN, BETHANY K. FULKERSON, JESSICA AMBROSE, ROBYN E. RICE, and RONALD L. WILEY

Physical Education, Health, and Sport Studies, Chemistry and Biochemistry, and Zoology Departments, Miami University, Oxford, OH 45056

ABSTRACT

ALESSIO, H. M., A. E. HAGERMAN, B. K. FULKERSON, J. AMBROSE, R. E. RICE, and R. L. WILEY. Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. Med. Sci. Sports Exerc., Vol. 32, No. 9, pp. 1576 –1581, 2000. Many studies have implicated elevated oxygen consumption (VO₂) associated with aerobic exercise as contributing to oxidative stress. Only a few studies have investigated nonaerobic exercise and its relation to pro-oxidant and antioxidant activities. Purpose: The purpose of this study was to compare biomarkers of oxidative stress: lipid peroxidation, protein oxidation, and total antioxidants in blood after exhaustive aerobic (AE) and nonaerobic isometric exercise (IE). Methods: Blood samples were collected from 12 subjects who performed a maximum AE and IE test and were analyzed for thiobarbituric acid (TBARS), carbonyls, lipid hydroperoxides (LH), and oxygen radical absorbance capacity (ORAC). Results: VO₂ increased 14-fold with AE compared with 2-fold with IE. Protein carbonyls increased 67% (P, 0.05) pre- to immediately and 1 h post-AE, and 12% pre- to immediately post-IE and returned to baseline 1 h post-IE. TBARS did not increase significantly with either treatment. LH increased 36% above rest during IE compared with 24% during AE (P, 0.05). ORAC increased 25% (P < 0.05) pre- to post-AE, compared with 9% (P < 0.05) pre- to post-IE. Conclusion: There was evidence of oxidative stress after both exhaustive aerobic and isometric exercise. Lipid hydroperoxides, protein carbonyls, and total antioxidants increased after both IE and AE. Due to the different metabolic demands of aerobic and isometric exercise, we can rule out a mass action effect of VO₂ as the sole mechanism for exercise-induced oxidative stress. Key Words: ANTIOXIDANT, PHYSICAL ACTIVITY, HAND GRIP, OXIDATIVE STRESS

Many studies have reported that physical exercise can induce oxidative stress (2,10,14,21,23). Most of these studies implicate aerobic exercise (AE) as the fundamental cause of elevated levels of oxygen centered free radicals (e.g., superoxide radicals: O₂⁻, hydroxyl radicals: OH⁻, hydroperoxy radical: HO₂⁻, and lipid peroxy radicals: LOO⁻). It has been suggested that a mass action effect initiated by elevated oxygen consumption (VO₂) generates abnormally high concentrations of oxygen free radicals. Usually, free radicals produced by the mitochondrial electron transport system are removed or scavenged by endogenous antioxidants (32). However, if free radical formation exceeds antioxidant capacity, the radicals may escape from the mitochondria and oxidize lipids, proteins, sugars, and other cell components. Lipid or protein oxidation to reactive intermediates (malondialdehyde or carbonyls, respectively) can alter or inhibit normal biochemical processes in the cell.

The magnitude of oxidative damage associated with exercise depends on the rate of VO₂, production of superoxide radicals, and the balance of antioxidant and pro-oxidant cellular mechanisms (15). An estimated 1–3% of total oxygen consumed results in formation of O₂⁻ (5). AE can increase whole body VO₂ from 10- to 20-fold over resting state (12). Given the high level of VO₂ accompanying AE, it is not surprising that biomarkers of oxidative stress increase in direct proportion to AE intensity. Most of the protocols used to study exercise-induced oxidative stress have used models with healthy individuals that participate in AE such as running or swimming with durations ranging from 20 min to exhaustion (2,18–20,34).

Isometric exercise (IE), like AE, when performed on a regular basis has been shown to lower resting blood pressure in hypertensive and borderline hypertensive subjects (36). The influence of IE on the formation of oxygen centered free radicals has been largely overlooked. If exercise-induced formation of O₂⁻ results mainly from elevated VO₂, then we would not expect significant oxidative stress to result from IE since it does not increase VO₂ significantly above the resting level. However, factors other than VO₂ can influence pro-oxidant and antioxidant status of cells. For example, stress hormones can undergo autoxidation with relatively low amounts of oxygen available (8,12). Heavy
metal ions such as iron (III) can catalyze oxidation reactions that cause radical formation (24). Other factors regulate exercise-induced oxidative stress, including level of fitness, type and intensity of exercise, and dietary antioxidants. The purpose of this study was to compare biomarkers of oxidative stress after exhaustive aerobic and isometric exercise (IE) in humans.

METHODS

Subjects. Twelve subjects (9 male, 3 female) volunteered for the study. Mean age was 25.2 ± 3.2, mean body weight was 88.7 ± 4.3 kg, mean VO$_{2\text{max}}$ was 3.53 ± 0.30 L·min$^{-1}$, and mean maximum grip force was 35.8 ± 2.9 kg. Experimental procedures were approved by the University Human Subjects Committee, and all subjects completed written informed consent forms before participating in the study.

Experimental protocol. Subjects completed a health history questionnaire on their first visit and a 2-d diet recall was completed on both visits to the laboratory to encourage and determine similar food intake. Subjects refrained from taking dietary or vitamin supplements during the study. The health history questionnaire revealed no significant findings that warranted dismissal from the study. The diet recall data were analyzed by “The Food Processor” software program (Esha Research, Salem OR), which included information on calories, food type and quantity, and antioxidant intake. Each subject performed a maximum AE test to exhaustion on a treadmill. Heart rate, blood pressure, expired gasses, rating of perceived effort, and total time of the test were recorded continuously. Immediately pre, immediately post, and 1 h post exercise, a blood sample was drawn from an antecubital vein and the same tests were repeated.

Biochemical analysis. Venous blood was collected into an evacuated tube, containing no anticoagulant. Samples for lactate concentration (0.050 mL) were immediately frozen at −80°C until analysis. Lipid hydroperoxides (LH) were measured according to Mihaljevic et al. (28) with chemicals purchased from Cayman Chemical Company (Ann Arbor, MI). Equal volumes of plasma and methanol were mixed. Then 1 mL of cold chloroform was added and mixed. After centrifuging at 1500 × g for 5 min at 0°C, the bottom chloroform layer was carefully transferred to another tube on ice. Absorbance of this layer was measured at 500 nm. The concentration of hydroperoxide values were calculated from a standard cumene hydroperoxide curve, corrected for volume, and expressed per mg serum protein determined with the BioRad assay.

Lipid peroxidation by-products were measured as MDA (11) with chemicals purchased from Calbiochem (San Diego, CA). First, 650 mL of 10.3 mM N-methyl-2-phenyldole, in acetonitrile, was added to 100-µL plasma and mixed. Then, 150 µL of 12 N HCl was added, mixed, and incubated at 45°C for 60 min. After cooling on ice, absorbance was measured at 586 nm and equivalents of malondialdehyde were calculated from a standard curve. Values were expressed per mg serum protein determined with the BioRad assay.

Protein carbonyls were measured by the method of Levine et al. (25). The proteins were precipitated from 50 µL aliquots of plasma with trichloroacetic acid and reacted for 60 min at room temperature with 2,4 dinitrophenyl hydrazine in 2 N HCl. The derivitized proteins were isolated by precipitation with trichloroacetic acid and washed extensively with a 1:1 solution of ethyl acetate:ethanol. The protein was redissolved in 6 M guanidine hydrochloride, and the absorbances at 364 nm were recorded and converted to molar quantities using the extinction coefficient, 22,000 M$^{-1}$ cm$^{-1}$, previously reported (25). Values were expressed per mg serum protein determined with the biocinchoninic acid method (Pierce Chemical Co., Rockford, IL) standardized with bovine serum albumin.

Lactate concentration was measured by a method previously described (13). Whole blood samples were initially collected in perchloric acid (50:1000). For the assay, 50 µL of sample was mixed with a 2000 µL of a buffer solution containing 100 mg NAD, 0.4 M hydrazine-0.5 M glycine buffer. After 60 min incubation at 45°C, fluorescence was measured with excitation = 360 nm and emission 460 nm. Values were expressed in mM·L$^{-1}$ according to a standard curve.

Antioxidant activity was indicated by the oxygen radical absorbance capacity (ORAC) in plasma according to an assay previously described (6). The ORAC assay is an
inhibition method in which a sample of serum is added to a free radical-generating system. The inhibition of the free radical action is measured and this inhibition is related to the antioxidant activity of the sample (7). In the ORAC assay system, 10 µL of plasma is used. The blood plasma was not deproteinized. Beta-phycoerythrin (b-pe) was used as a target for free radical attack by the free radical generator, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH). Serum, b-pe, and AAPH were mixed together in a potassium phosphate buffer, and the fluorescence of the b-pe was monitored on a fluorometer (excitation = 540 nm, emission = 575 nm) every 5 min until the emission of b-pe was zero (approximately 45–60 min later). The control standard was 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble vitamin E analog. Results are expressed as ORAC units×mL−1 plasma, where 1 ORAC unit equals the net protection produced by 1 µM Trolox per mL plasma.

Statistical analysis. Analysis of variance with repeated measures was used to compare means from oxidative stress biomarkers and lactate concentration after AE and IE. Sphericity was tested by the Greenhouse-Geisser method, and the Bonferroni correction was made to decrease the chance of a Type I error. Post hoc comparisons were made by comparison-contrast tests. A probability level of 0.05 was set for significance. We divided the original probability level of 0.05 by the number of ANOVAs (six) performed so that the 0.01 level of significance was set before we indicated a change occurred.

RESULTS

Table 1 reports mean data for selected nutrient components for 2-d dietary recall for the 12 subjects when they arrived for AE testing and for a week later when they arrived for IE testing. No significant differences in nutrient intake were observed between the two different exercise sessions.

Figure 1 reports VO2 changes during AE and IE to exhaustion. Figure 2 (a and b) shows heart rate and mean arterial pressure (MAP) responses, respectively, to exhaustive aerobic and isometric exercise. Both heart rate and MAP increased after either aerobic or isometric exercise. Compared with IE, heart rate increased more during exhaustive AE, but MAP increased more during IE. Ratings of perceived effort for AE and IE are given in Table 2. In Table 3 blood lactate responses to the different types of exercise are reported. Blood lactate concentration increased 479% after AE and 221% after IE. Pre-post differences were significant at P = 0.004, which was significant even with the Bonferroni correction to reduce the risk of Type I error. Table 4 includes the results from several measures of oxidative stress. There were no significant changes in plasma MDA pre-, immediately post-, and 1 h post-exhaustive AE and IE. In contrast, LH increased significantly after IE

<table>
<thead>
<tr>
<th>Total Daily Calories (Cal)</th>
<th>Percent Calories from Protein</th>
<th>Percent Calories from Carbohydrate</th>
<th>Percent Calories from Fat</th>
<th>Vitamin A (RE)</th>
<th>Vitamin C (mg)</th>
<th>Vitamin E (IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>2130</td>
<td>15.1</td>
<td>54.8</td>
<td>26.7</td>
<td>1429</td>
<td>175</td>
</tr>
<tr>
<td>(1308)</td>
<td>(5.7)</td>
<td>(11)</td>
<td>(14.3)</td>
<td>(705)</td>
<td>(125)</td>
<td>(4.6)</td>
</tr>
<tr>
<td>Week 2</td>
<td>2241</td>
<td>21.1</td>
<td>55.1</td>
<td>29.11</td>
<td>1237</td>
<td>141</td>
</tr>
<tr>
<td>(657)</td>
<td>(19.2)</td>
<td>(11)</td>
<td>(13.8)</td>
<td>(630)</td>
<td>(116)</td>
<td>(6.9)</td>
</tr>
</tbody>
</table>

Figure 1—Oxygen consumption changes during aerobic and isometric exercise to exhaustion.

Figure 2—Mean and SEM heart rate and mean arterial blood pressure responses to aerobic and isometric exercise. * Pre- and post-exercise values are significantly different, P < .01; ** isometric and aerobic exercise values are significantly different, P < .01.
(36.2% pre- to postmaximum IE, \( F = 4.77, P = 0.001 \). Table 4 also reports protein carbonyls in blood before, immediately after, and 1 h after aerobic and nonaerobic exercise. Carbonyls increased 67% immediately post maximum aerobic exercise (\( P = 0.01 \)) compared with 12% immediately post maximum isometric exercise. The antioxidant biomarker, ORAC, increased more after AE (25%, \( P = 0.01 \)) than after IE (9%, \( P > 0.01 \)) (Table 5). One hour post IE and AE, plasma ORAC levels remained elevated, but were not significantly different than rest (Table 5).

### DISCUSSION

Many studies have reported that acute aerobic exercise contributes to oxidative stress, especially when performed at high intensity levels (1,9,19). Two mechanisms linking acute aerobic exercise and oxidative stress are a) increased pro-oxidant activity via a mass action effect when \( \dot{V}O_2 \) is elevated 10- to 15-fold above rest, and 2) inadequate antioxidant activity relative to pro-oxidants. Further studies have reported that although acute AE may increase oxidative stress, chronic aerobic exercise may attenuate it (2,16,17,22) due to increased endogenous production of antioxidants and/or more tightly coupled electron transport system allowing fewer electrons to escape and form superoxide radicals. Consensus has not been reached in that yet another study (35) reported that acute aerobic exercise did not immediately result in increased oxidative stress, but they observed a gradual increase in resting urine MDA in the group that aerobically trained over 8 wk.

In the present study, acute AE resulted in a 14-fold increase in \( \dot{V}O_2 \) above rest. Carbonyls increased 67% pre to immediately post exercise. Antioxidant protection against pro-oxidant activity, indicated by ORAC\(_{ROO}^{-}\), increased 25% pre to immediately post AE. Results from this study concur with others that very intense, exhaustive AE tilts the balance from antioxidant toward pro-oxidant activity, giving rise to exercise-induced oxidative stress.

Few studies have investigated either the acute or chronic effects of nonaerobic exercise on biomarkers of oxidative stress, and no study has investigated isometric exercise and oxidative stress. In the present study, subjects performed IE until exhaustion. The protocol used in the present study controlled for exercise time so that workload IE was performed for a similar amount of time as the AE test. Levels of MDA were not affected by either exhaustive IE or AE. Levels of LH increased 36% (\( P < 0.01 \)) above rest during IE compared with 24% (\( P > 0.01 \)) during AE. A major distinction between IE and AE was the oxidation of proteins where exhaustive IE did not change carbonyls significantly (12%) compared with a 67% increase (\( P < 0.01 \)) after AE. The increase in antioxidant level (\( P < 0.01 \)) associated with AE was about twice the increase (\( P > 0.01 \)) with IE.

Pro-oxidants, including LIPOX by-products, have previously been reported to accumulate after nonaerobic exercise in mice. Alessio and Cutler (1) reported that 1 min of high-intensity running (45 m\( \text{min}^{-1} \)) resulted in 167% and 157% elevated TBARS in red slow-twitch and white fast-twitch muscle, respectively. LH increased 34% and 31% in red slow-twitch and white fast-twitch muscle, respectively. Because the high-intensity running lasted for only 1 min, it is likely that increased \( \dot{V}O_2 \) was not the exclusive mediating factor for TBARS or LH generation. A study by Ortenblad et al. (30) had subjects perform six bouts of 30-s strenuous jumping with 2-min rest between bouts. Biomarkers of LIPOX did not increase significantly, but several key antioxidants (e.g., superoxide dismutase, glutathione peroxidase, and glutathione reductase) did significantly increase. Higher antioxidant levels may have been partly responsible for attenuating LIPOX by-products. McBride et al. (29) studied the interactive effects of resistance training and vitamin E supplementation on MDA. Subjects completed heavy resistance exercise that consisted of three sets of 10

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**TABLE 2. Rating of perceived effort (Borg scale 6–20) to aerobic and isometric exercise.**

<table>
<thead>
<tr>
<th>Percent of Test Effort</th>
<th>Isometric</th>
<th>Aerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>20</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>40</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>60</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>80</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>18</td>
</tr>
</tbody>
</table>

**TABLE 3. Mean and standard error of the mean (in parentheses) blood lactate (mM L\(^{-1} \)) responses to the different types of exercise (\( N = 12 \)).**

<table>
<thead>
<tr>
<th>Exercise Type</th>
<th>Pre-Exercise</th>
<th>Immediately Post-Exercise</th>
<th>1 h Post-Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol·mg(^{-1} ) protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic exercise</td>
<td>0.14 (0.01)</td>
<td>0.16 (0.01)</td>
<td>0.14 (0.01)</td>
</tr>
<tr>
<td>Isometric exercise</td>
<td>0.14 (0.01)</td>
<td>0.15 (0.01)</td>
<td>0.14 (0.01)</td>
</tr>
<tr>
<td>Lipid hydroperoxides (nmol·mg(^{-1} ) protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic exercise</td>
<td>4.1 (0.4)</td>
<td>5.1 (0.6)</td>
<td>4.9 (0.4)</td>
</tr>
<tr>
<td>Isometric exercise</td>
<td>3.9 (0.5)</td>
<td>5.3 (0.6)*</td>
<td>5.2 (0.7)*</td>
</tr>
<tr>
<td>Protein carbonyls (nM·mg(^{-1} ) protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic exercise</td>
<td>2.2 (0.2)</td>
<td>3.6 (0.4)*</td>
<td>2.7 (0.3)</td>
</tr>
<tr>
<td>Isometric exercise</td>
<td>2.4 (0.2)</td>
<td>2.7 (0.2)</td>
<td>2.3 (0.2)</td>
</tr>
</tbody>
</table>

*Post-exercise is significantly different than pre-exercise, \( P \leq 0.01 \).
maximal repetitions for each of eight different exercises, allowing 2-min rest between sets. MDA increased up to three-fold above resting levels immediately, 6, and 24 h after one bout of these resistance exercises. Vitamin E supplementation attenuated MDA increase and was associated with a return of MDA to resting levels within 6 h post resistance exercise. Atalay et al. (4) conducted a sprint training study whereby rats performed sessions of 30-s bouts of intense sprinting. They were sacrificed at rest 48 h after their last sprint session and showed increased selected antioxidants in fast twitch and mixed skeletal muscle but not slow twitch or heart muscle. The few strength and sprint training studies on oxidative stress do not concur about the effects on pro-oxidant–antioxidant balance.

Although a mass action effect of oxygen consumption has usually been associated with oxidative stress reactions, including lipid and protein oxidation, it is also recognized that factors other than elevated oxygen consumption contribute to disturbances in metabolism and superoxide radical production. Oxygen-centered radicals have been observed during the autoxidation of catecholamines, hemoglobin, and thiols (8,12). Mechanisms for isometric exercise-induced oxidative stress may also include mechanical stress. Mechanical stress contributes to exercise-induced damage of muscle fibers, even in the absence of increased VO\(_2\). In particular, eccentric exercise, where the muscle generates high levels of force while lengthening, has been shown to initiate cytokine activity that controls inflammation (33). LIPOX by-products, including cyclooxygenase- and lipoxygenase-derived products, are potent vasoactive and chemotactic factors. It is likely that LIPOX reactions may play a causal role in inflammation. In a study by Saxton et al. (33), both eccentric and concentric arm and leg exercise were performed and muscle soreness peaked two days after eccentric exercise. Immediately after concentric leg exercise in Saxton’s study, protein carbonyl derivatives and serum creatine kinase activity increased, although TBARS did not.

In our study, statistical power was affected by the limited number of subjects. It is difficult to generalize from 12 subjects; nevertheless, the trends in this study provide insight into differences in oxidative stress between IE and AE. AE affected total antioxidant capacity as well as several measures of oxidative damage, but significant oxidative changes after IE included only lipid hydroperoxides. Several distinctions between IE and AE could contribute to the different magnitude of stress response in the two exercise modes. Less VO\(_2\) during IE compared with AE (Fig. 1), and attenuated changes in lactate and blood pressure after IE, are consistent with relatively low oxygen demand and, thus, less oxidative stress. Alternatively, because IE involved forearm muscles, compared with the leg, hip, and back muscles used during AE, amounts and concentrations of ROS generated are probably different in the two types of exercise.

The dramatic blood pressure changes observed during isometric exercise in the present study may represent significant blood flow changes that delineates ischemia-reperfusion. In ischemic-reperfused models, oxygen radicals can be generated upon reflow after vasocstriction (3). Mitochondrial respiration is an important source of oxygen radicals and, hence, a potential contributor to reperfusion injury (27,31). During a 50% MVC, and probably relatively early, blood flow into the limb is completely occluded. During the ensuing anaerobic effort, metabolite build-up may occur, which could contribute to changing the antioxidant/pro-oxidant balance. Further, during the surge in hyperemia immediately after release of the effort, the reperfusion of the ischemic tissue could also contribute to that balance. Because we did not directly measure blood flow, this hemodynamic mechanism is one speculation on a mechanism for isometric exercise-induced oxidative stress.

In this study, lipids were more likely to undergo peroxidation during exhaustive IE and proteins were more likely to be oxidized and form carbonyls during exhaustive AE. Compared with IE, AE was associated with an approximately 2.5-fold increase in peroxyl radical quenching activity. The stimulus for pro-oxidant and antioxidant activities must differ between AE and IE due to the drastically different metabolic demands. These data support that different types of oxidative stress are linked to AE and IE, although a mechanistic basis for this distinction is not clear.

REFERENCES


