

# Protein Modification Responds to Exercise Intensity and Antioxidant Supplementation

MANFRED LAMPRECHT<sup>1,2</sup>, KARL OETTL<sup>1</sup>, GUENTHER SCHWABERGER<sup>3</sup>, PETER HOFMANN<sup>4,5</sup>, and JOACHIM F. GREILBERGER<sup>1</sup>

<sup>1</sup>Institute of Physiological Chemistry, Medical University of Graz, Graz, AUSTRIA; <sup>2</sup>Styrian Health Association, Graz, AUSTRIA; <sup>3</sup>Institute of Physiology, Medical University of Graz, Graz, AUSTRIA; <sup>4</sup>Institute of Sports Science, Karl Franzens University of Graz, Graz, AUSTRIA; and <sup>5</sup>Human Performance Research Graz, Karl Franzens University and Medical University of Graz, Graz, AUSTRIA

## ABSTRACT

LAMPRECHT, M., K. OETTL, G. SCHWABERGER, P. HOFMANN, and J. F. GREILBERGER. Protein Modification Responds to Exercise Intensity and Antioxidant Supplementation. *Med. Sci. Sports Exerc.*, Vol. 41, No. 1, pp. 155–163, 2009. **Purpose:** To assess the effects of different exercise intensities and antioxidant supplementation on plasma protein modification. **Methods:** Trained men ( $n = 41$ ) from a homogenous population were randomly assigned to perform cycle ergometer exercise either at 70% or 80% of individual  $\dot{V}O_{2\max}$ . Each intensity group was randomly assigned to receive either juice powder concentrate (JPC 70%,  $n = 11$ ; JPC 80%,  $n = 10$ ) or placebo (Plac 70%,  $n = 10$ ; Plac 80%,  $n = 10$ ) capsules for 28 wk. Four controlled exercise bouts and blood collections were conducted at baseline and study weeks 4, 16, and 28. Blood samples were drawn before (BE), immediately after (IE), and 30 min (30M) and 30 h (30H) postexercise. These samples were analyzed to estimate concentrations of carbonyl groups on plasma proteins (CP) and the redox state of human serum albumin (HSA). **Results:** In the Plac group, CP concentrations increased at 80% of  $\dot{V}O_{2\max}$  IE and 30M, returning to preexercise concentrations by 30H ( $P < 0.05$ ). At both 16 and 28 wk, the Plac groups had significantly higher BE and 30H CP concentrations than the JPC groups ( $P < 0.05$ ). The reduced fraction of HSA, human mercaptalbumin (HMA), decreased at all four exercise tests at both exercise intensities IE and 30M, returning to preexercise values by 30H ( $P < 0.05$ ). Supplementation had no influence on HSA. **Conclusions:** These results indicate that CP concentrations increase with 80%  $\dot{V}O_{2\max}$  intensity. The JPC group had lower baseline CP levels after 16 and 28 wk and no exercise-induced CP increase. HSA is reversibly shifted to a more oxidized state by recent intense exercise. **Key Words:** ALBUMIN REDOX STATE, CARBOXYLATED PROTEINS, DEFINED EXERTION, DIETARY SUPPLEMENTS

Oxidative stress-related exercise studies and reviews report that physical exercise of sufficient intensity and duration can result in increased generation of reactive oxygen and nitrogen species (3,33). These are eliminated by the body's antioxidant system, including both endogenous compounds and exogenous nutritional substances, such as those found in plant foods. If the antioxidant defense is inadequate, oxidative processes dominate, resulting in "oxidative stress" that can lead to damaged lipids, proteins, carbohydrates, and DNA (6,27,33). In athletes, oxidative stress results in muscular oxidative damage (11), muscle soreness (30), loss of

skeletal muscle force production (16), or impaired immunity (8,25,26).

Several researchers report that exogenous antioxidant supplementation attenuates oxidative stress biomarkers such as carbonyl groups on protein (CP), malondialdehyde, or 8-hydroxydeoxyguanosine (3,9,18,34). However, there is also some evidence that high-dose antioxidant supplementation may increase lipid peroxidation after exhaustive exercise (15,24).

The redox state of human serum albumin (HSA) is a potential approach to investigate the extracellular redox state in exercise. This is the main protein in extracellular fluids, and its redox state appears to be influenced by physical exercise (13). This protein contains a single cysteine residue not involved in a disulfide bond. In HSA, cysteine-34 can exist in several forms: the reduced form with a free thiol group (human mercaptalbumin, HMA); a reversibly oxidized form, wherein cysteine-34 forms a disulfide with low molecular weight thiol compounds such as cysteine (human nonmercaptalbumin 1, HNA1); or a further oxidized form, as a sulfenic or sulfonic acid state (human nonmercaptalbumin 2, HNA2). The main fraction of serum albumin, HMA, is thought to participate in maintaining an

Address for correspondence: Manfred Lamprecht, Ph.D., Institute of Physiological Chemistry, Harrachgasse 21/II, Medical University of Graz, 8010 Graz, Austria; E-mail: manfred.lamprecht@meduni-graz.at.

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appropriate redox potential in blood or interstitial fluid. Although the oxidation step from HMA to HNA1 is readily reversible, the oxidation to HNA2 is less so (5). To our knowledge, this is the first study of the possible influence of defined exercise intensities or antioxidant treatment on HSA redox states.

Any effects of defined aerobic exercises during a long-term antioxidant supplementation on CP concentration or HSA redox state is not established. This study investigates these effects in a homogenous, trained, and compliant cohort of police special forces. We hypothesized that the extent of exertion and/or use of a commercially available encapsulated juice powder concentrate (JPC) might influence the concentrations of CP or the redox state of HSA in plasma of trained men. We evaluated the influence of defined cycle exercise bouts at 70% and 80% of individual maximum oxygen uptake ( $\dot{V}O_{2max}$ ) on these parameters and repeated the exercise test after 4, 16, and 28 wk of JPC or placebo (Plac) use.

## METHODS

**Subjects.** All subjects provided written informed consent before participation in this investigation. This study was conducted in compliance with the guidelines of the Declaration of Helsinki for Research on Human Subjects and was approved by the Human Ethics Committee at the Medical University of Graz, Austria. The study cohort consisted of 41 healthy men (nonsmokers, members of the police special forces ["Cobra"]) who volunteered to participate. All subjects completed a medical history, a diet analysis, and a physical activity questionnaire before the beginning of the investigation to determine eligibility. Exclusion criteria included use of tobacco products, chronic or excessive alcohol consumption, recent surgery or illness, and use of pharmaceuticals, drugs, or dietary supplements within the 4 wk before the study baseline visit. Medications (i.e., antibiotics) or injuries during the study period were documented, and the duration and time point of these events determined continued inclusion or exclusion from the study. Further determinants for eligibility included the requirement that subjects should have had trained aerobically at least 3 d·wk<sup>-1</sup> for a minimum of 1 yr before participation and had a minimum level of aerobic fitness as assessed with maximal testing ( $\dot{V}O_{2max} > 45$  mL·kg<sup>-1</sup>·min<sup>-1</sup>). Body fat content and distribution were estimated by a computerized optical device (Lipometer; Möller Messtechnik, Graz, Austria) to determine subcutaneous adipose tissue thickness (22).

**Study design.** The study was randomized, double-blind, and placebo controlled. Anthropometric measurements, first diet analysis, a first standard blood chemistry panel, and determination of  $\dot{V}O_{2max}$  were carried out 4 wk before the start of the experimental period. Subjects were randomly assigned, in a double-blind fashion, to either the

70% or the 80%  $\dot{V}O_{2max}$  group and then further divided to take either the JPC or the Plac capsules, resulting in four study groups. At study weeks 4, 16, and 28, exercise tests were repeated. All subjects were checked by a physician before each endurance test.

**Incremental exercise tests.** All subjects performed an incremental cycle ergometer exercise test (model no. "ERG 900S"; Schiller, Baar, Switzerland). After 3 min sitting on the ergometer (rest), work rate started at 40 W for 3 min and was increased by 20 W every minute until voluntary exhaustion. A standard 12-lead ECG was recorded during the entire physician-supervised test.

**Respiratory gas exchange.** Respiratory variables were measured throughout all tests using a facemask and breath-by-breath mode with data stored at 10-s intervals. Oxygen uptake, carbon dioxide output, minute ventilation, breathing rate, and tidal volume were continuously obtained using a portable open-air spirometry system (MetaMax I; Cortex Biophysik, Leipzig, Germany). Analyzers were calibrated before the tests, with gases of known concentration. Ventilatory threshold (VT<sub>2</sub>) was assessed using a computer-aided linear regression break point analysis (Prospert, Leitner, Austria).

Blood lactate concentration was determined enzymatically (Boehringer, Mannheim, Germany). Capillary blood samples were taken at rest, at the end of each load step, after 3 min of active recovery, and after 3 min of passive recovery. The first increase of blood lactate concentration above resting level was defined as the first lactate turn point (LTP<sub>1</sub>). The second abrupt increase of blood lactate concentration, around 4 mmol·L<sup>-1</sup>, was defined as the second lactate turn point (LTP<sub>2</sub>). Lactate turn points were assessed using a computer-aided linear regression break point analysis (Prospert).

HR was measured throughout all tests using a commercially available HR monitor (Polar Vantage NV; Polar Electro, Kempele, Finland). Data were measured and stored at 5-s intervals, transferred to a computer, and analyzed as described previously (12).

**70% or 80%  $\dot{V}O_{2max}$  exercise tests.** Exercise tests were performed at baseline and were repeated after 4, 16, and 28 wk of supplementation with assigned capsules. Similar to the maximal test, subjects completed a rest phase sitting on the ergometer for 3 min. Exercise started at 40 W with 80 rpm for 3 min, and work rate was increased by 20 W every minute until the target workload at 70% or 80% of individual  $\dot{V}O_{2max}$  was attained. Workload was adjusted every 5 min to maintain the target % $\dot{V}O_{2max}$  intensity. Gas exchange variables and HR were monitored continuously throughout the exercise test as described above. Blood lactate concentration was determined at rest, after every load step, and after each 5 min of constant load exercise until termination of the test. After 20 min of exercise, the facemask was removed briefly to allow consumption of 250 mL of plain water. Test duration was 40 min of exercise or after reaching subject exhaustion, defined as the

inability to maintain exercise at the given workload and at 80 rpm.

**Dietary assessment.** All subjects were instructed to maintain their habitual diet during the study period and to complete daily food records for 6 d before and 1 d after the first exercise test for nutrient intake assessment. Subjects subsequently received copies of their 7-d diet records and were instructed to replicate the diet before the next exercise tests to minimize any influence of dietary fluctuation on the markers being monitored. In addition, 3 h before each exercise test, a standardized breakfast was fed to the subjects providing about 4222 kJ, 32–34 g protein, 144–150 g carbohydrate, and 28–30 g fat to further minimize any fluctuation due to recent diet. Food records were analyzed twice (at baseline and after 28 wk for total calories, proteins, carbohydrates, fat, cholesterol, fiber, water, alcohol, and micronutrient content using “Opti Diet” software (GOEmbH, Linden, Germany).

**Study capsules.** Study subjects randomized to Plac ( $n = 20$ ) received capsules containing microcrystalline cellulose identical in appearance to JPC. Subjects randomized to JPC ( $n = 21$ ) received capsules containing primarily berry, fruit, and vegetable JPC (Juice Plus<sup>®</sup>; NSA, Collierville, TN). The JPC capsules provided approximately 7.5 mg  $\beta$ -carotene, 200 mg vitamin C, 60 mg vitamin E, 600  $\mu$ g folate, and about 63 kJ·d<sup>-1</sup>. Subjects took six capsules daily for 28 wk.

**Physical activity, duties, and illnesses.** Subjects performed two to three sessions of endurance and weight training per week in addition to special mission specific training. Intensity, duration, and frequency of training were recorded and summarized weekly. Each subject was instructed not to perform hard physical training 4 d before each exercise test and within 30 h after each test, until after the last blood sample was drawn. The unit commissioner documented all mission assignments, and the unit physician documented the types of missions, duty hours, incidents of illness, injury, and other stressors (i.e., circadian imbalance).

**Blood collection and sample preparation.** We conducted four blood collections per subject at each of the four exercise tests: at the beginning of each exercise test, before exercise (BE = resting values), immediately after the end of each exercise test (IE), 30 min (30 M), and 30 h after the tests (30 H). At these times, 600  $\mu$ L of capillary blood (EDTA-coated vials; Sarstedt, Graz, Austria) was collected to determine CP, HMA, HNA1, and HNA2. Further, erythrocyte antioxidant enzyme activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined. After centrifugation at 3000g for 10 min, plasma was removed and samples were stored frozen at  $-70^{\circ}\text{C}$  until analysis.

**Analysis of CP.** Measurement of CP was done with a sensitive chemiluminescent immunoassay (LUMIstar; BMG LABTECH, Offenburg, Germany). After derivatization with dinitrophenyl-hydrazine (DNPH), anti-DNPH-antibody

(Sigma, St. Louis, MO) was used on 96-well microtitration plates (Nunc, Roskilde, Denmark) as described elsewhere (20). Assessment of plasma protein concentration used the bicinchoninic assay (Pierce, Rockford, IL).

**Analysis of HMA, HNA1, and HNA2.** Albumin was fractionated using high-performance liquid chromatography to give three peaks corresponding to the cysteine-34 redox state: the free thiol form (HMA), as a mixed disulfide (HNA1), or more oxidized (HNA2), as previously described (14). Quantification was based on the peak heights of HSA fractions, and data are expressed as the percentage of HMA, HNA1, and HNA2.

Because the redox state of HSA is expressed as the fraction of HMA, HNA1, and HNA2, respectively, and CP values are related to total plasma protein content, possible dehydration due to exercise did not influence these parameters.

**Analysis of SOD and GPx activity.** Assessment of SOD activity used erythrocyte lysate with xanthine oxidase in the start reagent, as previously described (28) expressed in U·mg Hb<sup>-1</sup>. Determination of GPx activity from erythrocyte lysate was performed indirectly by a coupled reaction with glutathione reductase and used the ZeptoMetrix Corporation (Buffalo, NY) assay kit adapted to 96-well plates, with results expressed in U·g Hb<sup>-1</sup>.

**Blood chemistry panel.** Standard blood chemistry values were determined from 5 mL EDTA plasma at subject enrollment and the end of the investigation, except CK, which was measured at each blood collection. Analysis used routine methods and the clinical chemistry analyzer “Eurolyser” (Dia Team; Diagnostica und Arzneimittel Großhandel GmbH, Linz, Austria). Parameters monitored were glucose, choamoulesterol, triglycerides, creatine kinase, albumin, C-reactive protein, uric acid, lactate, and the liver enzymes lactate dehydrogenase and glutamate-pyruvate-transaminase. Assessment of hemoglobin and iron concentrations used the Advia clinical analyzer (Fa. Bayer, Leverkusen, Germany).

**Statistical analysis.** All statistical analyses were performed using the Statistical Package for the Social Sciences for Windows (version 12.0; SPSS Inc, Chicago, IL). Data are presented as mean  $\pm$  SD. Statistical significance was set at  $P < 0.05$ . Baseline characteristics, performance and respiratory data, and nutrient and clinical chemistry data were compared between groups using one-way ANOVA. Data obtained for CP, HMA, HNA1, HNA2, and for antioxidant enzymes were analyzed using a 2 (intensity)  $\times$  2 (treatment)  $\times$  4 (time) repeated-measures ANOVA for each exercise test at baseline and after 4, 16, and 28 wk of supplementation. At each time point (BE, IE, 30M, and 30H), HMA, HNA1, HNA2 values, CP concentrations, enzyme activities, and CK values were compared between the four groups by one-way ANOVA. If there was no influence of the grouping factor treatment, the two 70%  $\dot{V}O_{2\text{max}}$  groups and the two 80%  $\dot{V}O_{2\text{max}}$  groups were pooled at selected time points (for HMA, HNA1 at IE and 30M) and compared by Student’s *t*-test for unpaired

samples to evaluate differences due to exercise intensity. If there was no influence of the grouping factor intensity, the two JPC groups and the two Plac groups were pooled at selected time points (for CP at BE and 30H) and were compared by Student's *t*-test for unpaired samples to evaluate differences due to treatment. Pearson regression analysis and correlation coefficient were used to evaluate bivariate relationships for HMA, HNA1, and CP data.

## RESULTS

**Characteristics of the study population.** The four groups did not differ in age, height, weight, total body fat, lean body mass,  $\dot{V}O_{2\max}$ , maximum workload, or duty hours during the 28-wk study period ( $P > 0.1$ ; Table 1). From baseline to week 28, the amount of duty hours per month increased from ~1150 to ~1650 (approximately 40%) in all groups.

**Analysis of the 7-d food.** Analysis for daily kilojoule, macro- and micronutrient intake found no statistically significant differences between groups for any measured nutrient variable ( $P > 0.1$ , data not shown). Average food group servings were similar in all groups, although inadequate concerning fruit and nonstarchy vegetable intake averaging 3.2 servings per day ( $P > 0.1$ , data not shown). Baseline clinical blood chemistry parameters were equivalent in the groups and did not change throughout the study period. Capsule compliance was >85% in all four groups.

**Exercise data.**  $VT_2$  and  $LTP_2$  occurred at  $79 \pm 3.5\%$  and  $78.1 \pm 3.6\%$  of  $\dot{V}O_{2\max}$ , respectively, and were not significantly different between exercise groups ( $P > 0.1$ , Table 1). Seventy percent  $\dot{V}O_{2\max}$  exercise performance was  $230 \pm 15$  W,  $39.8 \pm 1.4$  min of duration, and at  $39.7 \pm 1.8$  mL·min<sup>-1</sup>·kg<sup>-1</sup> oxygen uptake. True intensity performed was at  $71.1 \pm 2.6\%$  of  $\dot{V}O_{2\max}$ . Eighty percent  $\dot{V}O_{2\max}$  performance was  $250 \pm 20$  W,  $37.6 \pm 4.3$  min of duration, and at  $44.2 \pm 2.9$  mL·min<sup>-1</sup>·kg<sup>-1</sup> oxygen uptake. True intensity performed was at  $78.3 \pm 3.4\%$  of  $\dot{V}O_{2\max}$ . Exercise in the 70%  $\dot{V}O_{2\max}$  groups was significantly below  $VT_2$  and/or  $LTP_2$  exercise intensity (~10%) reflecting blood lactate concentrations lower than threshold values (Table 1). Exercise in the 80%  $\dot{V}O_{2\max}$  groups was, on average, the

same as  $VT_2$  and/or  $LTP_2$  resulting in lactate concentrations significantly higher than the 70%  $\dot{V}O_{2\max}$  group values (Table 1). No significant differences between % $\dot{V}O_{2\max}$  groups were noted for duration of constant load exercise ( $P > 0.1$ ).

**Concentrations of CP.** Across all CP determinations, concentrations ranged from 0.21 to 0.99 nmol·mg protein<sup>-1</sup>. There were no differences between the four groups in CP concentrations BE and 30H at the baseline exercise test and after 4 wk ( $P > 0.1$ ). After 16 and 28 wk, CP concentrations were lower in the JPC groups than in the Plac groups BE and 30H ( $P = 0.017$  after 16 wk,  $P = 0.006$  after 28 wk). At baseline, we found an intensity  $\times$  time-dependent effect to higher concentrations at 80%  $\dot{V}O_{2\max}$  intensity compared with 70%  $\dot{V}O_{2\max}$  intensity ( $P = 0.021$ , Fig. 1A) IE and 30M. After 4, 16, and 28 wk, this increase was diminished in the 80% JPC group. In both JPC groups and in the 70% Plac group, CP concentrations did not change over the time course of the exercise tests after 4, 16, and 28 wk ( $P > 0.1$ ), only the 80% Plac group showed significantly increased CP concentrations IE and 30M ( $P < 0.05$ ; Figs. 1B–D). After 28 wk, the increase in CP concentrations in the 80% Plac group was more pronounced IE and 30M.

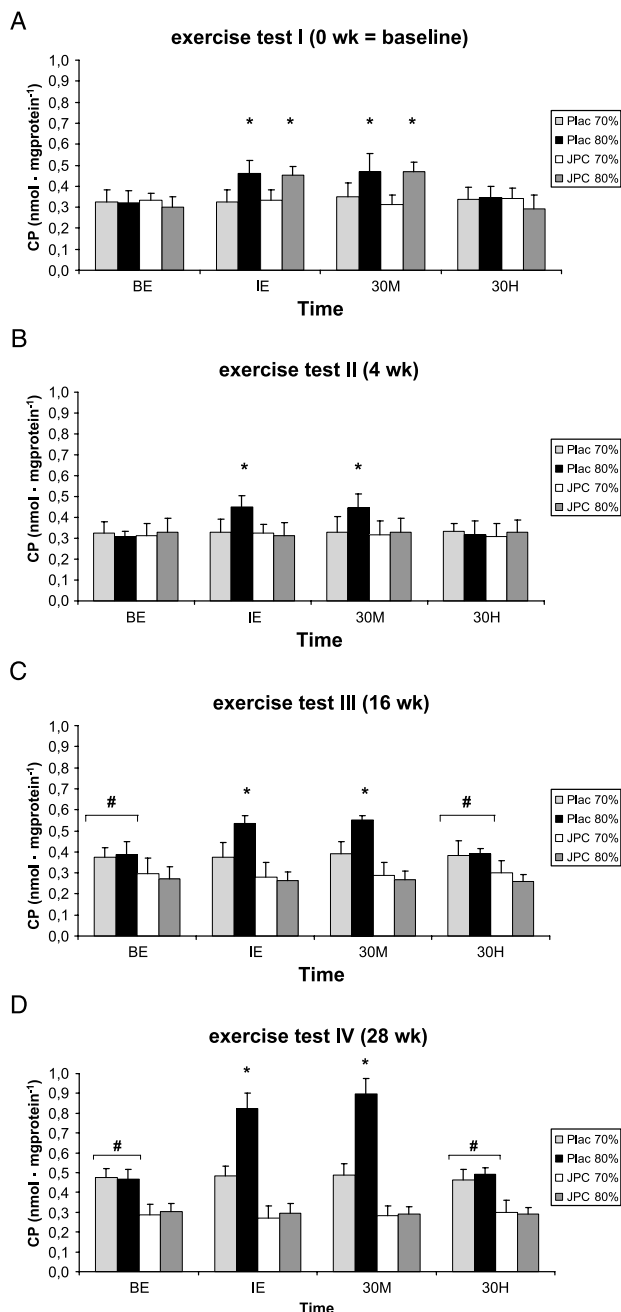
**Values of HMA.** Across all HMA determinations, percentages ranged from 61.1% to 73.2%. There were no differences between the four groups in HMA percentages BE and 30H at any exercise test ( $P > 0.1$ ). As shown in Figures 2A–D, there were lower HMA percentages IE and 30M, returning to preexercise values by 30H in all four groups at all four exercise tests ( $P < 0.05$ ). This effect was more pronounced at 80%  $\dot{V}O_{2\max}$  intensity compared with 70%  $\dot{V}O_{2\max}$  intensity at each test ( $P < 0.05$ ). Supplementation had no influence on HMA values at any exercise test ( $P > 0.1$ ).

**Values of HNA1.** Across all HNA1 determinations, percentages ranged from 24.4% to 38.3%. There were no differences between the four groups in HNA1 percentages BE and 30H at any exercise test ( $P > 0.1$ ). As shown in Figures 3A–D, there were increased HNA1 percentages IE and 30M, returning to preexercise values by 30H in all four groups at all exercise tests ( $P < 0.05$ ). This effect was more

TABLE 1. Characteristics of the 41 study participants.

Variable	JPC 70% (n = 11)	JPC 80% (n = 10)	Plac 70% (n = 10)	Plac 80% (n = 10)
Age, yr	34.3 $\pm$ 5.1	36.4 $\pm$ 3.8	33.8 $\pm$ 5.7	35.1 $\pm$ 4.2
Height, cm	183.2 $\pm$ 11.4	181.2 $\pm$ 7.4	180.8 $\pm$ 8.6	182.3 $\pm$ 9.4
Weight, kg	83.6 $\pm$ 8.1	81.7 $\pm$ 6.3	79.8 $\pm$ 5.4	82.5 $\pm$ 6.7
Total body fat, %	12.9 $\pm$ 3.8	11.9 $\pm$ 5.2	12.4 $\pm$ 4.2	13.6 $\pm$ 3.7
Lean body mass, kg	72.7 $\pm$ 6.6	71.2 $\pm$ 5.3	69.9 $\pm$ 4.7	70.8 $\pm$ 7.9
Max. workload, W	345 $\pm$ 35	340 $\pm$ 30	335 $\pm$ 25	340 $\pm$ 25
$\dot{V}O_{2\max}$ , mL·kg <sup>-1</sup> ·min <sup>-1</sup>	57.4 $\pm$ 7.7	56.4 $\pm$ 8.5	54.8 $\pm$ 9.3	55.8 $\pm$ 6.1
$VT_2$ , % of $\dot{V}O_{2\max}$	79.0 $\pm$ 3.8	77.9 $\pm$ 3.2	79.4 $\pm$ 3.1	79.7 $\pm$ 3.5
$LTP_2$ , % of $\dot{V}O_{2\max}$	78.1 $\pm$ 3.1	77.5 $\pm$ 2.9	77.8 $\pm$ 3.7	79.1 $\pm$ 4.0
Lactate during exercise, mMol·L <sup>-1</sup>	3.32 $\pm$ 0.81	5.73 $\pm$ 2.44*	3.21 $\pm$ 0.97	5.95 $\pm$ 2.68*
CK, U·L <sup>-1</sup> ; BE	227 $\pm$ 24	220 $\pm$ 44	219 $\pm$ 38	221 $\pm$ 46
CK, U·L <sup>-1</sup> ; 30H	226 $\pm$ 31	287 $\pm$ 28*	224 $\pm$ 33	294 $\pm$ 41*
Duty hours at baseline, h·month <sup>-1</sup>	1253	1139	1147	1129
Duty hours after 28 wk, h·month <sup>-1</sup>	1768	1613	1619	1591

Values are presented as means  $\pm$  SD except for duty hours (total amounts). Comparison of groups resulted in  $P > 0.1$ , except for lactate and CK: \*  $P < 0.05$  (one-way ANOVA).



**FIGURE 1**—Carbonyl groups on plasma proteins (CP) concentrations in nmol·mg protein<sup>-1</sup> in plasma, 3 h after a standardized breakfast, at 70% and 80% of individual  $\dot{V}O_{2\max}$  exercise intensity, with JPC or placebo (Plac) treatment. Plac 70%:  $n = 10$ ; Plac 80%:  $n = 10$ ; JPC 70%:  $n = 11$ ; JPC 80%:  $n = 10$ . Four time points of blood collection: BE = before exercise, IE = immediately after the exercise test, 30M = 30 min after the exercise test, 30H = 30 h after the exercise test. A = at baseline (0 wk), B = after 4 wk of JPC or Plac treatment, C = after 16 wk of JPC or Plac treatment, D = after 28 wk of JPC or Plac treatment. \*Significant effect of intensity  $\times$  time (A;  $P < 0.05$ ) or intensity  $\times$  treatment  $\times$  time (B, C, D;  $P < 0.05$ ) by repeated-measures ANOVA. #Significant effect of JPC treatment ( $P < 0.05$ ) compared with Plac groups by  $t$ -test.

pronounced at 80%  $\dot{V}O_{2\max}$  intensity compared with 70%  $\dot{V}O_{2\max}$  intensity ( $P < 0.05$ ), observable at the exercise tests at baseline, after 4 wk, and after 28 wk. The exercise test

after 16 wk did not show a significant difference in HNA1 change between the 70%  $\dot{V}O_{2\max}$  and the 80%  $\dot{V}O_{2\max}$  group IE and 30M ( $P > 0.1$ ). Supplementation had no influence on HNA1 values at any exercise test ( $P > 0.1$ ).

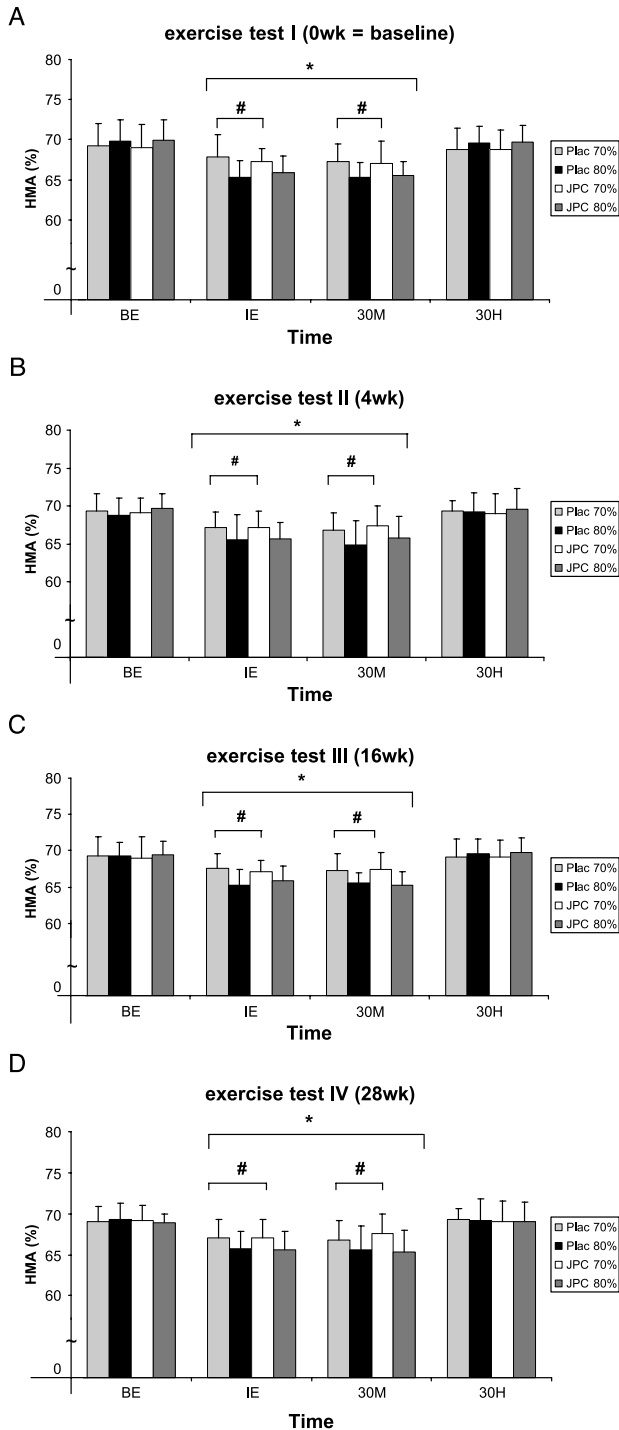
**Values of HNA2.** HNA2 percentages ranged from 1.6% to 3.1%. There were no differences between the four groups in HNA2 percentages at all time points and at any exercise test ( $P > 0.1$ , data not shown). Neither exercise intensity nor supplementation showed an effect on HNA2 values at any exercise test or time point of blood collection ( $P > 0.1$ ).

**SOD and GPx activities.** SOD activities were determined with a mean value of  $16.8 \pm 6.2$  U·mg Hb<sup>-1</sup>. GPx activities were determined with a mean value of  $212 \pm 46$  U·g Hb<sup>-1</sup>. There were no differences on these enzyme activities between the four groups at all time points and at any exercise test ( $P > 0.1$ , data not shown). Neither exercise intensity nor supplementation showed an effect on SOD and GPx values at any exercise test or time point of blood collection ( $P > 0.1$ ).

**Correlation analyses.** Inverse correlation was found when HMA values were compared with CP concentrations at 80%  $\dot{V}O_{2\max}$  at the baseline exercise test (JPC:  $P = 0.018$ ,  $r^2 = 0.961$ ; Plac:  $P = 0.020$ ,  $r^2 = 0.931$ ). Although HMA percentages decreased IE and 30M, CP concentrations increased at these time points. Both variables returned to baseline at 30H. This inverse correlation was also observed in the 80%  $\dot{V}O_{2\max}$  Plac group at the three subsequent exercise tests (4, 16, and 28 wk,  $P < 0.05$ ). A positive correlation was found in the JPC and the Plac group when HNA1 values were compared with CP concentrations at 80%  $\dot{V}O_{2\max}$  intensity at the baseline exercise tests ( $P = 0.011$ ,  $R = 0.998$ ): both variables increased IE and 30M, returning to preexercise values after 30H. This correlation was also observed in the 80%  $\dot{V}O_{2\max}$  Plac group for the 4-, 16-, and 28-wk exercise tests ( $P < 0.05$ ).

## DISCUSSION

Our main findings were as follows: 1) CP concentrations, as indicator of protein damage, increased at 80%  $\dot{V}O_{2\max}$  exercise intensity with 40-min duration; 2) the JPC treatment diminished this increase in CP at 4, 16, and 28 wk; 3) the JPC treatment decreased CP concentrations at BE and 30H at 16 and 28 wk; 4) the redox state of HSA was influenced by both exercise intensities: HMA percentage was reduced IE and 30M with recovery to near resting values by 30H postexercise and HNA1 showed the contrary course; 5) the shift of HSA from HMA to HNA1 IE and at 30M was significantly more pronounced in the 80%  $\dot{V}O_{2\max}$  groups; 6) HNA2 represents a minor fraction and the percentages did not change at any  $\dot{V}O_{2\max}$  intensity or test time point indicating no irreversible oxidation of HSA at cysteine-34 under these conditions; 7) the redox state of HSA was not affected by JPC treatment; and 8) at 80%  $\dot{V}O_{2\max}$  cycle exercise intensity, a correlation between the



**FIGURE 2**—Fraction of human mercaptalbumin (HMA) in plasma as percentage of serum albumin. See note for Figure 1. \*Significant effect of intensity  $\times$  time ( $P < 0.05$ ) by repeated-measures ANOVA. #Significant effect of intensity ( $P < 0.05$ ) between 70% and 80% groups by  $t$ -test.

reversible shift of HSA from HMA to HNA1 and CP was found at baseline and in the Plac group.

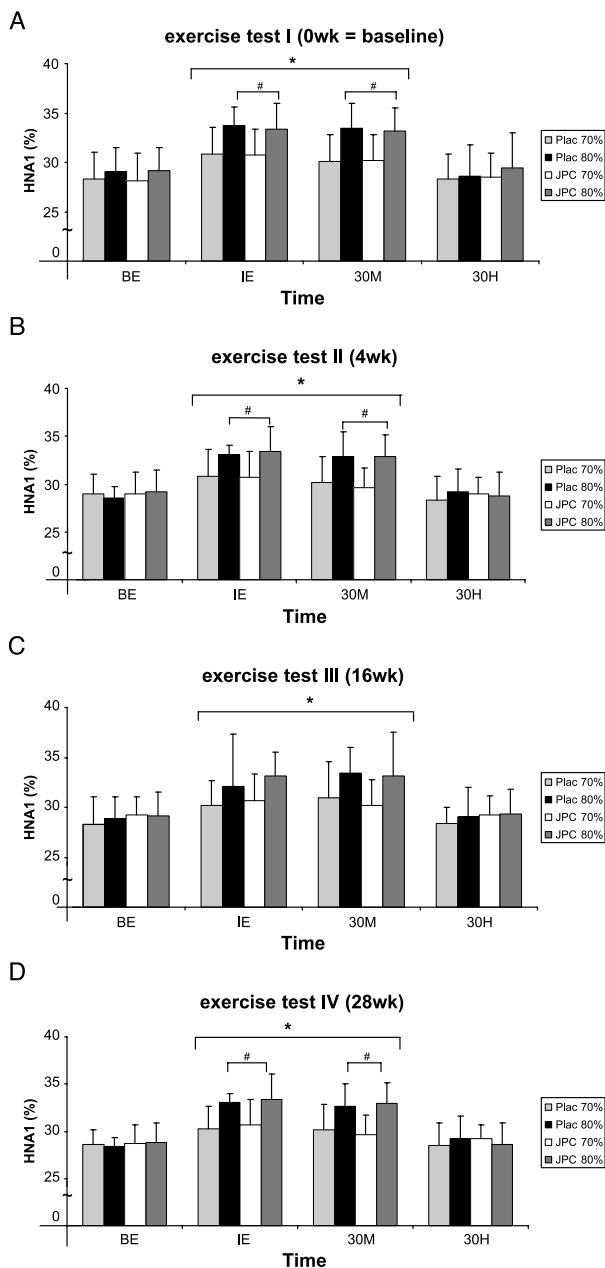
We hypothesized that the redox state of HSA and CP concentrations after intense exercise might be influenced by workload or intensity. Comparable studies have found

higher plasma CP values after aerobic exercises performed at 70% or 80%  $\dot{V}O_{2max}$  (2,3,10,27). In accordance with these studies, 70% and 80%  $\dot{V}O_{2max}$  intensities were chosen to ensure a detectable change in CP concentrations and to test our hypothesis on intensity-dependent changes in HSA redox fractions. Further, the second ventilatory turn point ( $VT_2$ ) and second lactate turn point ( $LTP_2$ ) in our subjects were estimated at 78–79% of individual  $\dot{V}O_{2max}$  (Table 1). Therefore, the 70% and the 80%  $\dot{V}O_{2max}$  were applied to induce a high exercise load in the subjects and to perform as close as possible to  $VT_2$  and  $LTP_2$  or about 10% below. Performing 40 min of exercise at the specified intensities was, on average, the longest possible duration at which subjects could perform at 80%  $\dot{V}O_{2max}$  to ensure supreme exertion together with the same exercise duration in both intensity groups.

An increase in CP concentrations was found at IE and 30M after 40 min exercise at 80%  $\dot{V}O_{2max}$ . At 70%  $\dot{V}O_{2max}$  exercise of similar duration, no increase in CP concentrations was observed in contrast to a study by Bloomer et al. (2). This apparent conflict could be due to the testing used to assess peak  $\dot{V}O_2$  values. Shorter protocols (8 min) could result in higher values than longer protocols (16 min), according to Yoon et al. (35). In our protocol, duration of the incremental step test was about 15–18 min on average, so the estimated peak  $\dot{V}O_2$  and consequently the  $\dot{V}O_{2max}$  percentages could have been lower for individual exertion than those reported by Bloomer (8–12 min). However,  $VT_2$  has been shown not to be affected by protocol duration (35) and was at 79% of  $\dot{V}O_{2max}$ , which was close to  $LTP_2$  (Table 1). This was also close to the true intensity performed by the 80% groups ( $78.3 \pm 3.4\%$ ). Our results confirm that oxidative protein damage can occur at exercise intensities close to  $LTP_2/VT_2$  for 40 min in trained men and can recover within 30 h.

The distribution of HSA fractions can be used as a systemic redox marker because albumin is the most abundant protein in plasma, is constantly exchanged between plasma and interstitial fluid, and is responsible for the largest fraction of reactive thiol groups (13). The main fraction of serum albumin, HMA, contributes to the maintenance of a constant redox potential, thus securing a certain redox buffer capacity in extracellular fluids (7). Thomas et al. (32) postulated that the oxidation of protein sulfhydryls to mixed disulfides and their reduction back to thiols might be an effective antioxidant system in extracellular fluids. Consequently, HMA might reflect an essential part of the redox buffer capacity in body fluids, especially in plasma. Therefore, HSA could become useful for oxidative stress studies in exercise, similar to other thiols like glutathione or lipoic acid (4,19,31). To our knowledge, this is the first study to investigate any influence of exercise with antioxidant supplementation on the redox state of serum albumin over several months in a fit population.

We found a significant shift to the disulfide form IE and 30M at all four exercise tests in all four groups. This effect



**FIGURE 3—**Fraction of reversible human nonmercaptalbumin (HNA1) in plasma as percentage of serum albumin. See note for Figure 1. \*Significant effect of intensity  $\times$  time ( $P < 0.05$ ) by repeated-measures ANOVA. #Significant effect of intensity ( $P < 0.05$ ) between 70% and 80% groups by  $t$ -test.

was more pronounced at 80%  $\dot{V}O_{2\max}$ . By 30H postexercise, the HMA fraction had returned to preexercise values regardless of exercise intensity the previous day. In contrast, HNA2 remained unchanged. These results indicate that intense exercise leads to a reversible disulfide formation on albumin's cysteine-34 and that irreversible oxidation did not take place. These data also show that 30 h or less are sufficient to return to the resting redox state of serum albumin. The specific model of exercise may have resulted in the direct utilization of albumin as an antioxidant during exercise. With higher intensity, greater utiliza-

tion appears. In this case, albumin is more a marker of redox status rather than a marker of oxidative stress. The results are in agreement with Imai et al. (13) who found significantly decreased HMA fractions after a 5-d Kendo training camp.

Albumin makes up approximately 55% of the total serum protein content and, combined with nine other abundant proteins, accounts for more than 90% of all serum proteins (1). Therefore, some researchers postulate that the increased concentration of CP after exercise should be mainly derived from the oxidation of albumin and other major serum proteins (19,21). If we compare HSA responses at the different exercise intensities to CP responses, we observe that HMA is shifted to HNA1 at both exercise intensities, whereas CP concentrations only increased at 80%  $\dot{V}O_{2\max}$ . This supports the idea that the oxidation of protein thiols to mixed disulfides may be an early response to oxidative stress, as postulated by others (32). The HSA redox buffer system seems to act at the beginning of the radical scavenging chain in plasma ("first line of defense"), whereas CP concentrations represent the end products of overwhelmed plasma antioxidant systems.

These data do not allow an estimate of the degree albumin contributes to the postexercise CP increase. This would require analysis of all abundant plasma proteins for CP content. The differences in the responses to supplementation and exercise found for albumin redox state and CP, respectively, emphasize different underlying mechanisms. The antioxidant supplemented JPC groups should have experienced the same increase in formation of reactive oxygen and nitrogen species due to exercise as the Plac groups. The HSA antioxidant system was active in all capsule groups, as represented by the shift of HMA to HNA1. However, in the JPC subjects, the antioxidant system was sufficient to prevent protein damage represented by the CP concentrations. Currently, we can only hypothesize why antioxidant supplementation did not influence the HSA redox system but avoided protein damage: the provided supplement might have acted somewhere in the radical chain reaction cascade between the HSA redox system ("first line of defense") and the end products of reactive oxygen and nitrogen species (RONS) attacks on proteins indicated via CP, a lack that needs to be investigated in future.

Beside the reduction of protein oxidation IE and 30M after 80%  $\dot{V}O_{2\max}$  exercise, the JPC groups had attenuated CP concentrations BE and 30H after 16 and 28 wk of supplementation. This was opposite to the Plac groups, which showed higher CP concentrations BE and 30H after these periods. The reduction of plasma CP concentrations in response to JPC treatment might be due to antioxidant bioavailability and increased antioxidant capacity in plasma as reported by others (29,23). The increase in CP in the Plac groups and the significant higher concentrations BE and 30H compared with the JPC groups after 16 and 28 wk indicate an increase in radical load at that time leading to

enhanced protein oxidation. Interestingly, this increase could be prevented by supplementation. The reason for the higher radical load is speculative but could be attributed to a higher amount of duty hours and to the increase in aircraft body-guarding duties (circadian imbalance) in all groups. Duty hours increased from ~1150 h at baseline to ~1650 h after 28 wk; about 10 h more duty per week for each subject, indicating an enhanced stress profile. Stress response resembles a pro-oxidative state with the potential for free radical damage to many cells and tissues (23).

The increase of duty hours was due to the Austrian chairmanship of the European Union from January to June 2006. This period represents study weeks 8 to 28 in the experimental period. Changes in antioxidant enzymes have not been detected during the whole study period.

Experience from our own determinations as well as from other groups (3,10) over the past years shows evidence that CP levels at rest within 0.2–0.4 nmol·mg protein<sup>-1</sup> can be regarded as normal range. Toward the end of the study, the Plac group had higher CP concentrations than 0.4 nmol·mg protein<sup>-1</sup>. Detrimental consequences, for example, on immune function or inflammation as published recently cannot be ruled out (17).

In conclusion, the results of this study indicate that HSA can act as a redox system in plasma under strenuous

exercise conditions. It was affected by exercise in an intensity-dependent manner, but not by exogenous antioxidants. It seems that the HSA redox system acts as an early response at the beginning of the radical scavenging chain. The HMA response to exercise will require further investigation to explore the potential of this biomarker as a valuable redox sensor, for example, to avoid protein damage induced by oxidative stress in exercise or to steer training load and recovery. Plasma CP concentration, as indicator of oxidative protein damage, increased significantly at intensities close to LTP<sub>2</sub>/VT<sub>2</sub>, and this increase was avoided in the JPC groups. The JPC groups had stable CP concentrations during increased duty hours.

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