Physiol. Res. 59: 953-961, 2010

Differential Impact of Acute Bout of Exercise on Redox- and Oxidative Damage-Related Profiles Between Untrained Subjects and Amateur Runners

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Received August 31, 2009
Accepted March 18, 2010
On-line June 9, 2010

Summary

Despite the demonstrated exercise-induced increase in reactive oxygen species (ROS) production, growing epidemiological evidence indicates that habitual, moderate physical activity reduces the incidence of several oxidative stress-based diseases. This apparent paradox can be explained taking into account that ROS produced during repeated exercise bouts may act as mild stressors able to trigger physiological and biomolecular hormetic responses through a number of redox-sensitive transcription pathways. Unfortunately, much more limited information is available from general population-based research, which could better reflect the condition of common people interested in achieving and maintaining good fitness levels. The present work aimed at investigating whether and how exercise-related habits in non-professional regular runners (n=33) can affect the systemic anti-oxidative capacity, and the resting serum levels of typical lipid peroxidation-related by-products and oxidatively-damaged proteins, in comparison with untrained sedentary individuals (n=25). We also analyzed in both groups the redox response elicited by a modified Bruce-based maximal exercise test on the same parameters. Our findings indicated that long-term regular and moderate practice of aerobic physical activity can increase antioxidant defense systems, lower the resting protein oxidation processes and reduce the immediate up-regulation of lipid-targeting oxidative stress in response to an acute bout of exercise.

Key words

Oxidative stress • Aerobic exercise • Physical fitness • Reactive Oxygen Species • Lipid peroxidation

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Introduction

Exercise is known to promote reactive oxygen species (ROS) production in humans and animals, mainly through increased leakage of oxygen-centered free radicals from the mitochondrial electron transport chain, ischemia-reperfusion-induced activation of xanthine oxidase, NAD(P)H-dependent superoxide release from activated neutrophils (Deaton and Marlin 2003).

ROS-based chemical attacks can lead to molecular damages and to oxidative stress, thereby cells possess highly conserved defense systems fulfilled by multiple interactions of antioxidant compounds, antioxidant enzymes, damage-removal and repair enzymes (König *et al*. 2001).

Despite the increased ROS production during

exercise, growing evidence derived from epidemiological and prospective studies strongly indicates that habitual, moderate physical activity reduces the incidence of oxidative stress-based diseases and retard the aging process (Ji *et al*. 2006, Laukkanen *et al.* 2009). This apparent paradox can be explained taking into account that reactive oxygen species produced during repeated exercise bouts could serve as mild stimulating stressors able to trigger hormetic responses. Organic adaptations can involve changes in cardiorespiratory and muscular physiology, the activation of redox-sensitive transcription pathways and the induction of the major endogenous defense systems. Indeed, Knez *et al*. (2006) and Radák *et al*. (2008) demonstrated that exercise training triggers adaptations in body's antioxidant defense systems. Significant differences have been reported in erythrocyte antioxidant enzymatic contents and in resting levels of blood antioxidant activities between trained and untrained individuals (Fielding and Meydani 1997, Urso and Clarkson 2003). Moreover, Santos-Silva *et al*. (2001) demonstrated that the training status influences not only the antioxidant capacity but also the oxidative molecular damage degree. On the other hand, physical inactivity is associated with physiological dysfunctions and reduced whole-body resistance to oxidative stress (for review see Radak *et al*. 2008). Nonetheless, a substantial debate still exists on this argument, mainly due to controversial or conflicting findings reported by some authors (Hubner-Wozniak *et al*. 1994, Tauler *et al*. 1999).

In addition, most of the existing studies on humans analyzed the oxidative stress profile in professional athletes or sportsmen habitual to high-intensity agonistic competitions (Gomez-Cabrera *et al*. 2006, Knez *et al*. 2006, Kostaropoulos *et al*. 2006, Skenderi *et al*. 2008). Much more limited seems to be the general population-based research which could better reflect the situation of common people interested in achieving and maintaining good fitness levels for health purposes.

On this basis, we designed a general population-based study in order to clarify whether and how the training status can affect the systemic anti-oxidative defense in untrained and trained healthy males that experienced a modified Bruce-based maximal exercise test. We paid a particular attention to the effect elicited by an ergometric test on the major oxidative damage marker profile. In particular, we measured serum thiobarbituric acid-reactive substances (TBARS) levels and protein carbonyl content (PCC), these compounds being widely used to evaluate in biological samples the complex processes of lipid peroxidation and oxidative damage to proteins, respectively (Levine *et al*. 1994, Yagi 1998). Serum total antioxidant capacities (TAC) were also assessed in order to estimate the cumulative action of high and low molecular weight antioxidants which cooperate in determining the overall reactive oxygen species removal rate in tissues and body fluids (Rice-Evans and Miller 1994).

Methods

Human subjects

Untrained sedentary subjects (n=25) and habitual aerobic male runners (n=33) participated to the study. All individuals needed to fulfill the following inclusion criteria: (i) absence of any acute or chronic inflammatory disease, (ii) absence of any metabolic disease including diabetes mellitus of any type, (iii) no medical history of hypertension, (iv) no clinical evidence of cardiovascular or peripheral artery disease, (v) no thyroid dysfunction, (vi) no concomitant medication intake, (vii) no alcohol, nicotine, or drug abuse. Severe overweight or obesity cases were excluded in the preliminary phase of the research. Untrained individuals were not engaged to any regular structured exercise activity and did not have physically demanding jobs (e.g. manual labor). Trained subjects presented similar running training histories (8±2 years, 4±1 h/week, 30±5 km/week). Participants were excluded if they had used vitamin or mineral supplements four weeks prior to the study. Furthermore, any participation in intense physical efforts was forbidden during the four weeks prior to the laboratory test. The experimental protocol was approved by the ethical committee of the University "G. d'Annunzio" of Chieti-Pescara and all participants provided their written, informed consent before the study. All the subjects were not allowed to take anti-inflammatory drugs within two days before the blood withdrawals. This work was conducted in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Main anthropometrical measurements of enrolled individuals are reported in Table 1.

Cardiopulmonary exercise testing

All participants underwent a physician-supervised maximal treadmill test, according to the modified Bruce protocol. Briefly, the test included seven stages (3 min each) with increasing speeds (2.74-8.05

km/h) and grades (0-18 %) of the treadmill.

All the tests were performed in the morning hours. Pre-exercise blood sample withdrawals were taken after a 12-h fasting period, in order to avoid digestion-induced interferences with the assessments. Two hours before the testing session, all subjects were fed 200 ml pear juice and two slices of bread. Ventilation, oxygen uptake and carbon dioxide output were measured by a computer-controlled breath-by-breath analyzer (Schiller CS-200 Ergo-Spiro), combined with an electrocardiograph for heart rate and rhythm control. Maximal oxygen uptake (VO_2max), was determined on the basis of the spirometric analysis of VO_2 and of VCO_2. Predicted maximal heart rate and anaerobic threshold were determined by using the 220 – age and V-slope methods, respectively. Lean body mass percentage was measured by standard impedentiometry-based procedures. Post-exercise blood withdrawals were taken 30 min after the maximal test. Isolated serum specimen was immediately frozen at –80 °C until processed. Laboratory temperature and humidity conditions were kept 20-23 °C and 50-55 %, respectively. During the exercise protocol, liquid consumption was not allowed.

Table 1. Main anthropometric parameters of the participants.

	Untrained (n=25)		Trained (n=33)		
	Mean	**S.D.**	**Mean**	**S.D.**	
Age (yrs)	39	3	42	1	
BMI (kg/m^2)	26.1	1.1	23.5	0.5	**
LBM (%)	80	2	86	1	**
VO_2max (ml/kg/min)	33.3	1.2	48.5	0.9	***
AT (ml/kg/min)	18.6	0.9	32.2	1.3	***
HR (bpm)	66	4	52.1	1.5	**
DAP (mm Hg)	75	2	74	4	
SAP (mm Hg)	116	2	119	4	

Values are given as means ± standard deviations (S.D.). BMI, body mass index; LBM, lean body mass; VO_2max, maximal oxygen consumption; AT, anaerobic threshold; HR, resting heart rate; DAP, resting diastolic arterial pressure; SAP, resting systolic arterial pressure. ** P<0.01, *** P<0.001 vs untrained (t-test for independent samples).

Total antioxidant capacity

Total antioxidant capacity was assessed by using the colorimetric 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)-equivalent antioxidant capacity (TEAC) assay kit (cat. 709001; Cayman Chemical, Ann Arbor, USA), which is based on the suppression of the absorbance of radical cations of 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS®) by antioxidants in the sample when ABTS is incubated with a peroxidase (metmyoglobin) and hydrogen peroxide (Rice-Evans and Miller 1994). Color development were read by a Victor3 microplate reader (Perkin Elmer, Waltham, USA).

Thiobarbituric acid-reactive substances

The measurement of thiobarbituric acid-reactive substances is a method commonly used in order to detect lipid peroxidation (Yagi 1998). We employed the TBARS Assay Kit (cat. 10009055; Cayman Chemical) which allows a rapid photometric detection of the thiobarbituric acid-malondialdehyde (TBA-MDA) adduct. Processed samples were read by a Lambda25 spectrophotometer (Perkin Elmer).

Protein carbonyls

Cayman Chemical's Protein Carbonyl Assay Kit (cat. 10005020) was used in order to evaluate colorimetrically oxidized proteins (Levine *et al.* 1994). Serum sample were treated as recommended by the manufacturer and the hydrazone-containing pellets were redissolved in guanidine hydrochloride. The color development was followed by a Victor3 microplate reader (PerkinElmer). The obtained values were normalized to the total protein concentration in the final pellets (absorbance reading at 280 nm), in order to consider protein loss during the washing steps, as suggested in the kit's user manual.

Statistical analysis

SigmaStat and Statistica 7 softwares were used for data processing. Values are given as means ± S.D. Two-way ANOVA for repeated measure (RM)-based analyses were performed in order to reveal the effects derived from life style (untrained vs. trained), graded exercise testing (pre vs. post) and interactions between the two factors. ANOVA RM were also used to assess anaerobic threshold-dependent statistical effect in the acute exercise bout-induced thiobarbituric acid-reactive substances response. Holm-Sidak and Newman-Keuls

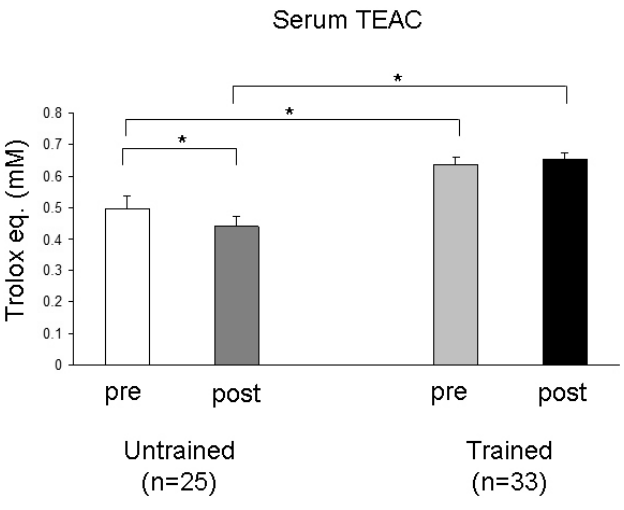

Fig. 1. Trolox-equivalent antioxidant capacity (TEAC) in serum of untrained subjects and age-matched amateur runners before and after a maximal treadmill-based ergometric test. Results are presented as means ± S.D. * P<0.05 Two-Way ANOVA for Repeated Measures followed by Holm-Sidak's Multiple Comparison post-hoc test.

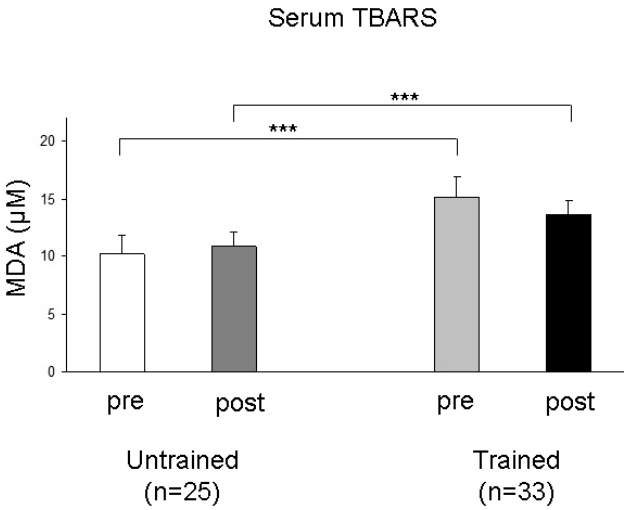

Fig. 2. Thiobarbituric acid-reactive substances (TBARS) levels in serum of untrained subjects and age-matched amateur runners before and after a maximal treadmill-based ergometric test. Results are presented as means ± S.D. *** P<0.001 Two-Way ANOVA for Repeated Measures followed by Holm-Sidak's Multiple Comparison post-hoc test.

multiple comparison *post-hoc* tests were applied when appropriate. Correlation studies were carried out by using the Pearson product moment method. Student's t-test for independent samples was used to compare anthropometrical measurements between the two groups. P<0.05 values were considered statistically significant.

Results

Anthropometric measurements comparison of untrained and trained individuals

The direct comparison of the anthropometric measurements in the two experimental groups revealed that trained subjects showed significantly higher VO_2max and anaerobic threshold (P<0.001) (Table 1). Coherently, trained participants had significantly lower body mass indexes and resting heart rates, as compared to untrained subjects (P<0.01). Trained individuals exhibited also higher lean body mass percentages, with respect to the sedentary group (P<0.01).

Cardiopulmonary exercise bout-induced effects (post vs pre comparisons)

No significant hemoconcentration was observed in subjects after the cardiopulmonary test (44.2±1.4 vs. 44.7±1.5).

Our statistical analyses showed that the cardiopulmonary exercise bout decreased significantly (P<0.05) Trolox-equivalent antioxidant capacity only in sedentary group, whereas in trained subjects the post-exercise total antioxidant capacity did not change, as compared to the pre-exercise condition (Fig. 1). The acute bout of exercise did not alter significantly any other individual redox-related parameter studied, as shown in Figures 1-3.

Life style-induced effects (untrained vs trained comparisons)

Our ANOVA-based statistical analysis revealed that trained subjects showed significantly higher levels of total antioxidant capacity, with respect to untrained individuals (P<0.05), both in pre- and post-exercise conditions (Fig. 1). In addition, the trained group exhibited significantly higher levels of lipid peroxidative damage, with respect to sedentary subjects (P<0.001), in both pre- and post-exercise samples (Fig. 2). On the contrary, trained individuals showed significantly lower protein carbonyl content, as compared to the untrained group (P<0.05), both in pre- and post-exercise samples (Fig. 3).

Dependence of acute exercise bout-induced changes in redox-related parameters upon aerobic performance levels

Significant relationships were revealed by correlation analysis between anaerobic threshold and cardiopulmonary exercise bout-induced variations of serum thiobarbituric acid-reactive substances levels, both

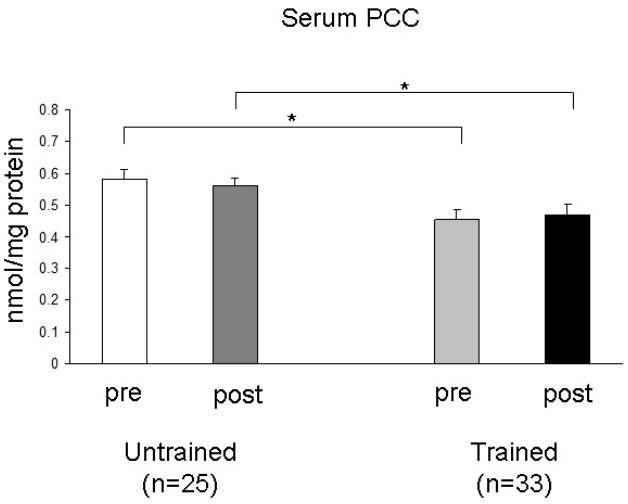

Fig. 3. Protein carbonyl content (PCC) levels in serum of untrained subjects and age-matched amateur runners before and after a maximal treadmill-based ergometric test. Results are presented as means ± S.D. * P<0.05 Two-Way ANOVA for Repeated Measures followed by Holm-Sidak's Multiple Comparison post-hoc test.

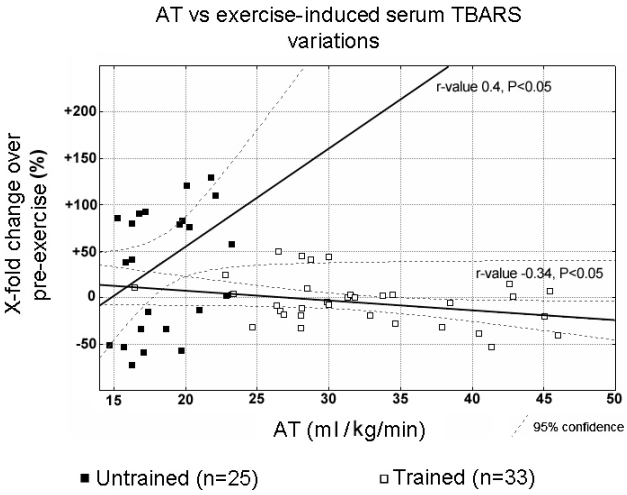

Fig. 4. Pearson product-moment correlation analysis between anaerobic threshold (AT) and thiobarbituric acid-reactive substances (TBARS) X-fold change (%) in serum of untrained subjects and age-matched amateur runners before and after a maximal treadmill-based ergometric test. The continuous and dotted lines show the linear regression function and the 95 % confidence interval, respectively.

in untrained and trained subjects (Fig. 4). In detail, untrained individuals showed a positive association between anaerobic threshold and post vs. pre TBARS change ($r = 0.40$, $P<0.05$), whereas in trained group these two parameters showed a significant negative correlation ($r = –0.34$, $P<0.05$). This finding led us to analyze more in depth a potential dependence of acute exercise-induced changes in TBARS concentrations upon the individual level of aerobic performances. The anaerobic threshold selected for the analysis represented the mean anaerobic threshold values ± standard deviations found in our groups. As shown in Figure 5, this investigation revealed that sedentary subjects with high anaerobic threshold (> mean + S.D.) showed an average increase of 40 % in serum thiobarbituric acid-reactive substances levels after the modified Bruce-based ergometric test ($P<0.05$), whereas in trained individuals characterized by high anaerobic threshold (> mean + S.D.), the acute bout of physical exercise caused a significant reduction of the lipid peroxidative damage marker ($P<0.01$).

No other significant relationships were found in correlation analyses.

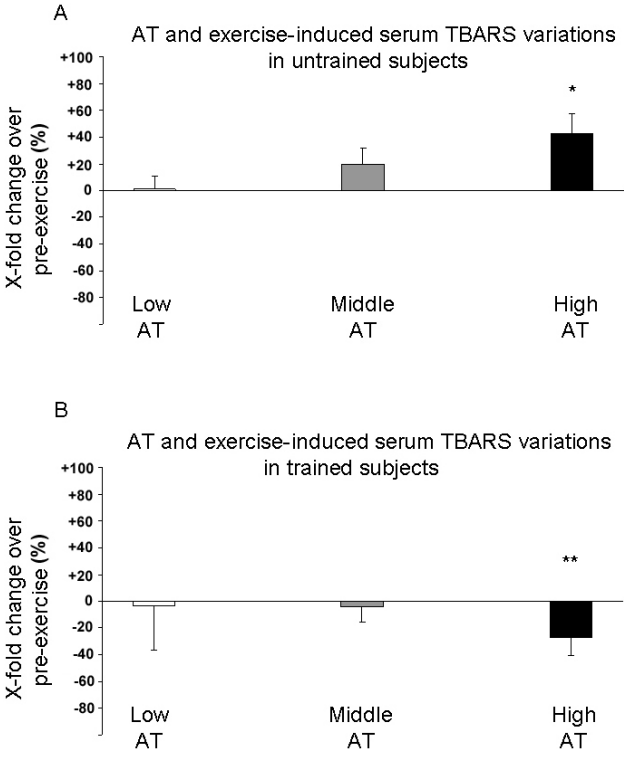

Fig. 5. Maximal ergometric test-induced variations (%) of serum thiobarbituric acid-reactive substances (TBARS) as a function of anaerobic thresholds (AT) in untrained subjects and age-matched amateur runners. Panel A, Untrained subjects: Low AT, anaerobic threshold less that mean - S.D. (n=5); Middle AT, anaerobic threshold within mean ± S.D. (n=16); High AT, anaerobic threshold more than mean + S.D. (n=4). Panel B, Trained subjects: Low AT, anaerobic threshold less than mean – S.D. (n=5); Middle AT, anaerobic threshold within mean ± S.D. (n=21); High AT, anaerobic threshold more than mean + S.D. (n=7).* P<0.05, ** P<0.01 vs pre-exercise (Two-Way ANOVA RM with Newman-Keuls Multiple Comparison post-hoc test).

Discussion

Epidemiological studies suggest that regular and moderate physical exercise reduces the incidence of oxidative stress-based diseases (Ji *et al.* 2006, Laukkanen *et al.* 2009). However, general population-based research

has not yet fully understood how exercise practice can affect redox homeostasis and alter systemic resistance against oxidative challenges. The present study was aimed at clarifying whether and how regular exercise practice may modify anti-oxidative defenses in the serum of untrained individuals and amateur runners that underwent a modified Bruce-based maximal exercise, with particular attention to the profile relative to major oxidative damage markers.

As expected, the anthropometric parameters of the two experimental groups significantly differed, as shown by the higher oxygen uptake and reduced resting heart rate found in trained subjects.

In order to verify that the observed results are independent of the differences observed for BMI between the two studied groups, we performed a Pearson product moment-based correlation analysis. This revealed that BMI does not significantly correlate to any parameter studied; untrained subjects' BMI were uncorrelated ($P>0.05$) to anaerobic threshold ($r = –0.394$), TBARS (pre-exercise, $r = 0.177$; post-exercise, $r = –0.305$), protein carbonyl content (pre-exercise, $r = 0.073$; post-exercise, $r = 0.077$), Trolox-equivalent antioxidant capacity (pre-exercise, $r = 0.388$; post-exercise, $r = 0.381$). Similar results ($P>0.05$) were revealed by analyzing trained subjects' BMI correlations to anaerobic threshold ($r = 0.069$), TBARS (pre-exercise, $r = 0.201$; post-exercise, $r = 0.242$), protein carbonyl content (pre-exercise, $r = 0.167$; post-exercise, $r = 0.188$), Trolox-equivalent antioxidant capacity (pre-exercise, $r = 0.039$; post-exercise, $r = –0.009$). This result seems to suggest that BMI cannot alter significantly individual profiles in relation to the other assessments examined in this study. A further confirm to this conclusion derived from BMI-adjusted data analysis. Indeed, all the presented results remained significant after data adjustment for individual BMI. These investigations strongly indicated that all the observed results do not dependent upon the differences observed for BMI between the two studied groups.

Trained individuals exhibited higher basal TEAC, with respect to untrained subjects, thus demonstrating that the training status is able to enhance the systemic antioxidant capacity even in non-professional exercisers. Our findings are coherent with those published by Shing *et al*. (2007), who reported enhanced resting plasma total antioxidant capacity in individuals exposed to repeated exercise bouts. A similar result was recently reported by Bloomer and Fisher-Wellman (2009), who observed higher TEAC values in trained smokers, as compared to age-matched untrained subjects. These data corroborate the hypothesis that regular exercise-induced ROS generation can trigger the activation of several redox-sensitive transcription factors with the consequent up-regulation of several antioxidant enzymes, such as the manganese-dependent superoxide dismutase (Ji 2008). This, in turn, may up-regulate antioxidant defense, thus leading to the improvements of aerobic individual performance. Coherently with this hypothesis, we and other researchers have previously demonstrated that aerobic performance is linked to circulating total or enzymatic antioxidant activity (Franzoni *et al*. 2004, Kostaropoulos *et al*. 2006, Falone *et al*. 2009). In particular, our latest findings could give a clue to Kostaropoulos' questions regarding the effect of chronic endurance exercise on total antioxidant status in human subjects.

Trained subjects showed significantly lower basal levels of circulating PCC, with respect to untrained individuals. Other researchers have found lower systemic PCC in subjects habitual to structured exercise (Bloomer and Fisher-Wellman 2008). Oxidative modifications can cause the loss of structural properties and catalytic functions in the targeted proteins and elevated concentrations of oxidatively-modified proteins have been linked to increased risk of various diseases, such as cancer, diabetes and cardiovascular disease (Dalle-Donne *et al*. 2003). People who regularly perform aerobic exercise are often exposed to ROS and this could induce molecular adaptations as improved removal rates of oxidatively-modified proteins, thus leading to enhanced resistance to further oxidative challenge. Accordingly, some researchers suggested that the proteasome complex could play a crucial role in the regular exercise-induced enhancement of the removal mechanisms of oxidized proteins (Ogonovszky *et al*. 2006, Radák *et al*. 2008). This sheds further light on how regular aerobic exercise could help people in preventing the development of several important syndromes and pathologies.

Lipid peroxidation (LPO) has been established as a major pathogenetic mechanism of cellular injury in humans (Halliwell 1991). Our study revealed that the trained group showed higher levels of circulating lipid peroxidation markers, as compared to untrained individuals; this result, apparently counterintuitive, is coherent to several reports published by others (Balakrishnan and Anuradha 1998, Santos-Silva *et al*. 2001, Gougoura *et al*. 2007, Shin *et al*. 2008, Mergener *et al*. 2009). Although LPO is causally linked to structural

and functional disturbance of biomembranes and has been mechanistically implicated in numerous disorders and diseases, recent *in vitro* studies suggested a potential role of lipid peroxidation products as regulators and modulators of cellular signaling and gene expression (Niki 2009). Nagy *et al*. (1998) demonstrated that oxidized lipids can interact with receptors for peroxisomal proliferators, known activators of antioxidant enzymes, such as catalase (CAT) and superoxide dismutase (SOD) (Gumieniczek *et al*. 2008). Moreover, pretreatment of cells with various LPO products at sublethal level improves cellular tolerance against forthcoming pro-oxidant attacks (Niki 2009). In agreement with considerations of Meilhac *et al*. (2000), we hypothesize that the presence of elevated steady-state levels of lipid peroxidation in the serum of habitual runners may be explained by taking into account that some lipid peroxidation-derived molecules could be required to induce and sustain the activation of redox-sensitive signaling pathways and, as a consequence, the elevation of overall antioxidant capacity. Similarly, higher concentrations of lipid peroxidation by-products could contribute to enhance protein catabolism through the ubiquitin-proteasome pathway (Gomes-Marcondes and Tisdale 2002).

Our findings revealed that subjects unfamiliar to physical activity were more susceptible to alterations of redox homeostasis caused by the acute bout of exercise, as shown by the reduction of the Trolox-equivalent antioxidant capacity observed in the untrained group after the ergometric test. On the contrary, our results showed that physical training is associated with beneficial adaptive responses which prevented the decline of TEAC following the acute exercise bout.

We did not find acute exercise-dependent changes in circulating protein carbonyl content. Only few studies regarding blood PCC following exercise are currently available, especially when considering human subjects. Alessio *et al*. (2000) reported increased protein carbonyl derivative levels immediately following exhaustive exercise. It is possible that aerobic exercise of a longer duration could produce protein carbonyl derivatives at greater concentrations. Indeed, elevations in protein oxidation markers are often exercise-induced retarded effects, since they strongly depend on the infiltration of phagocytic cells and on the potential loss in calcium homeostasis secondary to muscle injury (Bloomer *et al*. 2005).

Although we did not show any evident ergometric test-induced variation of circulating thiobarbituric acid-reactive substances, correlation analyses demonstrated a differential impact of the acute exercise on lipid peroxidation-derived by-products concentrations in untrained subjects and amateur runners. In detail, high anaerobic threshold-untrained subjects showed treadmill test-induced marked elevations of TBARS levels, as observed earlier (Jammes *et al*. 2004, Nikolaidis *et al*. 2007), whereas in high anaerobic threshold-trained individuals the ergometric run significantly lowered serum thiobarbituric acid-reactive substances concentration. Subjects with lower anaerobic threshold showed only slight modifications of systemic TBARS levels and this could be due to the shorter time required to achieve the maximum individual work load. Results presented here confirmed our previous findings regarding a negative association between endurance runners' aerobic performances and post-acute exercise increases in blood malondialdehyde concentrations (Falone *et al*. 2009). As well, our findings extended to moderate aerobic training support the conclusions of Shing *et al*. (2007), who demonstrated that consecutive days of high-intensity exercise enhanced resting plasma total antioxidant capacity and reduced the post-exercise increase in blood malondialdehyde concentrations. Our results seem to indicate that habitual exercise may be able to induce adaptations in removal efficiencies of lipid peroxidation by-products, thus reinforcing suggestions of Orhan *et al*. (2004) regarding a positive relationship between training status and urinary excretion rates of some small thiobarbituric acid-reactive substances, such as malondialdehyde (MDA).

The diverse thiobarbituric acid-reactive substances and protein carbonyl content change profiles observed after the ergometric test should be due to the extremely different turnover properties exhibited by TBARS (within minutes) and PCC (many hours to days), as suggested by Davies and Goldberg (1987).

In conclusion, the present study showed that long-term regular and moderate practice of aerobic physical activity could increase antioxidant defense systems and reduce the immediate up-regulation of lipid-targeting oxidative stress in response to an acute bout of exercise. Future research will try to elucidate in depth the molecular mechanisms involved in the training-induced adaptations which we presented in this work.

Conflict of Interest

There is no conflict of interest.

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