Oxidation of human serum albumin exhibits inter-individual variability after an ultra-marathon mountain race

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Received January 6, 2017; Accepted March 14, 2017

DOI: 10.3892/etm.2017.4268

Abstract. The aim of this study was to examine the oxidation of human serum albumin (HSA) caused by oxidative stress following exhaustive and demanding exercise, such as an ultra-marathon race. For this purpose, blood samples from 12 adult runners who underwent a 103 km mountain ultra-marathon race were collected before the race, and also at 24, 48 and 72 h post-race. HSA was partially purified using affinity chromatography and consequently subjected to western blot analysis in order to determine the levels of disulfide dimers indicating oxidation. For reasons of comparison, the results were correlated with those from a previous study, in which the same samples were analyzed using different oxidative stress markers. The results revealed a good correlation between albumin dimers and protein carbonyls at all time points, while there was also a significant correlation with static oxidation reduction potential at 24 h, and a negative correlation with capacity oxidation reduction potential at 24 and 48 h. In addition, an individual analysis of albumin dimers exhibited great inter-individual differences, indicating the variation of HSA oxidation between different athletes. Namely, in some athletes, HSA seemed to be the main oxidation target of serum proteins, while in other athletes, there was even a reduction of HSA. This inter-individual variability in the oxidation of HSA may suggest that different interventions (e.g., through diet) may be required in order to confront the effects on athletes following strenuous exercise. On the whole, this study suggests the importance of the assessment of albumin dimers as a predictive marker for exercise-induced oxidative stress.

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Abbreviations: CAT, catalase; EDTA, ethylenediamine tetraacetic acid; GSH, glutathione; HSA, human serum albumin; PC, protein carbonyls; ROS, reactive oxygen species; sORP, static oxidation-reduction potential; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances

Key words: albumin, oxidative stress, exercise, ultra-marathon mountain race, protein carbonyls

Introduction

The association between physical exercise and the increase in the production of free radicals has been well established (1,2). Free radicals are products of normal metabolism and include mainly reactive oxygen species (ROS), such as superoxide radical ($O2•^-$), hydroxyl radical (OH•), and peroxyl radical (RO2•), as well as reactive nitrogen species (RNS), such as nitric oxide (NO) and the peroxynitrite radical (ONOO•) (3). The excessive production of ROS may affect several cell functions, such as the regulation of signaling pathways and is involved in gene expression and apoptosis (4). ROS generation is affected by endogenous sources, such as the mitochondrial respiratory chain, inflammation and cytochrome P450 activity (5), or exogenous sources, such as smoking, air pollution and UV light (6). However, ROS generation during exercise and in particular during aerobic exercise, is believed to be caused by the increased uptake of oxygen from the active peripheral skeletal muscle tissues (2). Excessive ROS production may lead to a pathological condition known as oxidative stress (7). For the determination of oxidative stress levels following exercise, a number of oxidative stress markers are assessed, such as the levels and activity of antioxidant enzymes and molecules, oxidative DNA damage, lipid peroxidation and protein oxidation (8-12).

As regards protein oxidation, this is usually assessed by measuring protein carbonyl (PC) levels in plasma. The most abundant protein in plasma (approximately 50% of total protein) is human serum albumin (HSA) (13,14). HSA is a multifunctional, non-glycosylated globular protein composed of 585 amino acids with a molecular weight of 66 kDa, and is mainly synthesized in the liver (15-17). The structure of the

protein contains a center made up of hydrophobic radicals used as a binding site for ligands, while the outer part is composed of hydrophilic ligands. More specifically, albumin binds to and transfers several ligands, such as bilirubin, hormones, metal ions and xenobiotics (18).

In addition, HSA possesses a free thiol group in Cys34, and thus it may function as an extracellular antioxidant by scavenging ROS (19,20). Davies and co-workers published a series of studies explaining in detail the association between oxidative damage and the increased proteolytic susceptibility of bovine serum albumin (BSA) (21-24). Particularly, albumin residues contain cysteine and methionine sulfhydryl groups reacting with peroxides, leading to thiol oxidation (25,26). It is considered that albumin acts as an antioxidant, since Cys34 of albumin, a cysteine that represents approximately 80% of the total thiol content in plasma, scavenges ROS (27). However, under oxidative stress conditions, albumin is oxidized and Cys34 forms a disulfide with low molecular weight thiols, such as cysteine. Thus, the oxidation caused by free radicals may affect the molecule's conformation and structure (28). Moreover, albumin dimers have been reported as products of peroxidation caused by free radicals, and consequently they may be used as a marker of oxidative stress (29). Furthermore, since a number of studies have reported the association between the oxidation of albumin and exercise (20,28,30), the determination of albumin dimer levels may be a good indicator of oxidative stress in athletes.

Therefore, the present study focused on the determination of the levels of albumin dimers in the plasma of runners participating in an exhaustive mountain marathon race, 'Olympus Mythical Trail 2015'. This mountain marathon race covers a distance of 103 km in the mountain of Olympus in Northern Greece. It is considered to be one of the most demanding routes worldwide, since it includes a 7,200 m elevation gain and a highest altitude of 2,906 m, while 40 km of the route take place at an altitude higher than 2,000 m.

Moreover, in one of our previous studies, protein oxidation in the plasma of these runners was determined spectrophotometrically by phosphatidylcholine assay in order to assess the redox status from 24 to 72 h post-race (31). Thus, we also examined the correlation between the levels of HSA oxidation with the PC levels of total plasma protein, as well as with other oxidative stress markers. The determination of these correlations would help to determine whether HSA oxidation is a good marker for the assessment of oxidative stress following exercise.

Materials and methods

Subjects. Twelve adult male runners aged 41.1±3.2 years, voluntarily participated in the present study (height, 1.78±0.02 m; weight 72.9±2.0 kg). The subjects were informed not to receive any anti-inflammatory medicines or nutritional supplements and they were all familiar with mountain running.

The participants visited the Litohoro Health Center, located close to the starting point, 8 h before the race in order to complete a health and activity questionnaire, and their anthropometric parameters were taken. Moreover, written informed consent to participate in the study was provided by all the participants prior to blood collection. Body mass was measured to the nearest 0.5 kg (beam balance 710; Seca, Birmingham, UK) with the subjects lightly dressed and barefoot. Standing height was measured to the nearest 0.5 cm (Stadiometer 208; Seca).

All the performed procedures were in accordance with the Helsinki declaration of 1975 as revised in 2000 and approval was received by the human subjects committee of the University of Thessaly.

Description of the race. The volunteers participated at one of the most extreme mountain ultra-marathons worldwide known as the 'Olympus Mythical Trail', on July 4-5th 2015 in Olympus Mountain in Northern Greece. The peculiarity and difficulty of the race lies on the fact that it is a 103 km 'loop'-type route with a total ascent (positive height difference) of 7,200 m (more than twice the altitude of Olympus Mountain), while approximately 40 km of the route are at an altitute above 2,000 m. The starting and ending points are placed at Litohoro town in Greece. The route consists mostly of paths (95%) and dirt (5%) and is divided into 18 checkpoints. The maximum time allowed for race completion was 28 h.

Subject performance. Following the completion of the race, 8 out of 12 participants managed to finish the race (individuals no. 1, 2, 3, 4, 5, 6, 9 and 11), while 2 of them gave up at the 70th km (individuals no. 7 and 10) and the other 2 at the 60th km (individuals no. 8 and 12). The mean running time of the athletes was 19.57±1.09 h.

Blood collection and processing. The blood samples (10 ml) were drawn from a forearm vein with the subjects in the seated position at four different time points; 8 h before the competition (pre-race sample) and at 24, 48 and 72 h post-race. The samples were stored in ethylenediamine acid (EDTA) tubes and centrifuged at 1,370 x g for 10 min at 4˚C to divide the erythrocytes from the plasma. The plasma lysates were then stored at -80˚C prior to biochemical analysis.

Albumin determination assay. Albumin was determined spectrophotometrically at 628 nm, based on the formation of a coloured complex with bromocresol green reagent (BCG) solution in a 0.075 M succinate buffer (pH 4.20) (32).

Partial purification of albumin. For sample preparation, 1 volume of plasma was diluted in 50 volumes of a 0.1 M HEPES buffer (pH 7.0), containing 1 mM EDTA (Buffer A). The column was equilibrated by 10 ml of Buffer A using an AKTA prime protein purification system (AKTA purifier UPC 10; GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and the diluted sample was then applied to a Blue Sepharose column (1 ml) (HiTrap Blue HP; GE Healthcare Bio-Sciences AB). The column was washed with 10 ml of Buffer A, and the purified HSA was eluted and selected in a test tube using a 10 ml solution of 0,15 M KCl containing Buffer A (Buffer B).

Western blot analysis of albumin and albumin dimers. Protein concentration in plasma following the purification of the samples was measured using the Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA). The calculation of the albumin concen-

tration was made by using an albumin standard curve. Albumin monomers and dimers were then determined in the purified plasma samples by western blot analysis using a non-reducing SDS loading buffer. A non-reducing SDS loading buffer was used, as non-reducing conditions allow the visualization of any disulfide-linked dimers (33). Specifically, non-reducing buffers do not contain 2-mercaptoethanol (2-ME) or dithiothreitol (DTT), which can reduce disulphide bridges in proteins. In order to perform western blot analysis, the purified sample was diluted until the final concentration of 1 µg of albumin was achieved. Afterwards, an aliquot containing the diluted purified sample and a 2X non-reducing loading buffer was prepared, heated in boiling water for 3 min and separated by SDS-PAGE, using a polyacrylamide gel 8% (w/v). After 1 h of electrophoresis at 150 V, proteins were transferred onto polyvinylidenedifluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA) and blocked overnight with 5% non-fat milk in TBST (13 mmol/l Tris, 150 mmol/l NaCl (pH 7.5) solution, containing also 0.2% Tween-20 (TBSTMS).

The membranes were then incubated in a shaker for 1 h at room temperature with a goat anti-human albumin antibody diluted 1:5,000 in TBSTMS. Following extensive washes in TBST (5 times for 5 min) the blots were incubated for 30 min with anti-goat IgG secondary antibody (1:3,000 dilution). The membranes washed in TBST (3 times for 15 min) and the labeled protein bands were visualized by enhanced chemiluminescence (Bio-Rad Laboratories) and subsequent exposure to XAR 5 film (Fujifilm Corp., Tokyo, Japan). The protein bands were quantified using Alpha View quantification software (Alpha Innotech, San Leandro, CA, USA). Each sample was analyzed in triplicate.

Statistical analysis. The statistical analysis was based on one-way ANOVA followed by Dunnett's test for multiple pairwise comparisons. The statistical significance level was set at P<0.05. Correlations between oxidized albumin and the other oxidative stress markers were examined by Spearman's correlation analysis. The level of significance was also set at P<0.05. For all statistical analyses, SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA) was used. Data are presented as the means ± standard error of the mean.

Results

Western blot analysis was used for the assessment of albumin monomers and dimers. Monomer bands were displayed at ~55 kDa, while dimer formation appeared at approximately 110 kDa (Fig. 1). The lower molecular weight of the dimer bands compared to the theoretical one has been observed previously (29).

In each sample, the percentage ratio of dimers to monomers was quantified and considered as marker of HSA oxidation. The percentage change of oxidized HSA (i.e., the ratio of dimers to monomers) at 24, 48 and 72 h post-race compared to pre-race is shown in Fig. 2. There were not statistically significant differences between time points post-race and pre-race (Fig. 2).

Similarly, the percentage ratio of dimers to the total amount of HSA [i.e., dimers/(dimers + monomers)] was also quantified in order to obtain a clearer view regarding the changes of the protein after the race (Table I). Also, in this case, there were no statistically significant differences in the values post-race compared to those pre-race.

Table I. Percentage of dimer HSA to total HSA of athletes at all time points.

	Oxidized/total HSA levels (%)			
	Time point (h)			
Individual	Pre-race	24	48	72
1	36.59	34.12	30.19	27.42
2	39.06	34.27	37.31	44.01
3	45.37	44.84	38.53	29.22
4	28.96	37.98	30.38	37.12
5	36.43	31.74	28.09	22.88
6	36.58	36.04	43.06	34.82
7	38.38	35.12	33.47	39.18
8	21.95	22.92	25.51	24.46
9	25.44	24.68	27.93	15.42
10	39.51	41.73	40.26	39.90
11	30.11	32.19	36.51	37.55
12	35.11	33.60	32.74	35.95
Mean	34.95±2.02	34.60±1.89	34.17±1.36	32.83±2.53

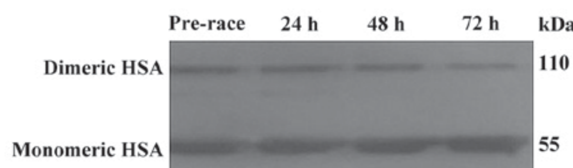

Figure 1. Representative image for the detection of monomeric and dimeric serum albumin (HSA) by western blot analysis. Specifically, it is shown the HSA from individual no. 2 at all time points (pre-race and 24, 48 and 72 h post-race). Albumin was obtained by Blue sepharose column chromatography and subjected to SDS-PAGE 8% (w/v) gel, under non-reducing conditions prior to immunoblotting analysis.

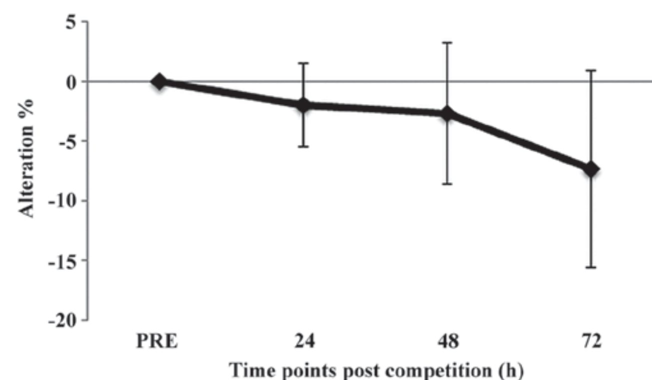

Figure 2. Percentage alteration of oxidized HSA (i.e., dimers/monomers ratio) in the plasma samples from the athletes participating in the mountain marathon race at 24, 48 and 72 h post-race compared with pre-race (PRE) samples. The values indicate the means ± standard error of the mean.

Table II. Correlation analysis between percentage changes of HSA oxidation at 24, 48 and 72 h post-race compared to pre-race and the corresponding percentage changes of PC, TBARS, GSH, sORP and cORP oxidative stress markers.

	Time point (h)	PC	TBARS	GSH	TAC	CAT	sORP	cORP
HSA	24	0.769[a]	-0.329	0.448	-0.098	-0.147	0.58[a]	-0.601[a]
	48	0.867[b]	0.336	0.063	-0.329	-0.231	0.448	-0.657[a]
	72	0.860[b]	-0.091	-0.140	-0.007	-0.524	-0.105	0.056

[a]Significantly correlation (P<0.05). [b]Significantly correlation (P<0.01). PC, protein carbonyls; TBARS, thiobarbituric acid reactive species; GSH, glutathione; TAC, total antioxidant capacity; CAT, catalase activity; sORP, static oxidation-reduction potential; cORP, capacity oxidation reduction potential.

In a previous study, in the samples, we assessed the percentage changes at 24, 48 and 72 h post-race in the oxidative stress markers, namely PC levels, thiobarbituric acid reactive species (TBARS) levels, glutathione (GSH) levels, total antioxidant capacity (TAC), catalase (CAT) activity, static oxidation-reduction potential (sORP) and capacity oxidation-reduction potential (cORP) compared to those at pre-race (31). In order to determine whether the changes in HSA oxidation are associated with any other marker, a correlation analysis was carried out between the percentage changes of HSA at time points post-race and the percentage changes of the other oxidative stress biomarkers (Table II). The results revealed that there was a significantly high correlation between HSA oxidation and PC at all three time points post-race (Table II). There were also moderate, yet significant correlations between HSA oxidation and sORP at 24 h post-race, as well as with cORP at 24 and 48 h post-race (Table II).

In our previous study, we demonstrated that there were great differences in the percentage changes post-race of oxidative stress markers (including PC) between different individuals (31). Thus, it was suggested that some markers should be examined individually in order to make safer conclusions and apply the appropriate interventions (25). Similarly, the changes in HSA oxidation (i.e., dimer/monomer percentage) post-race were also examined individually (Figs. 3 and 4). Moreover, since HSA exhibited a high correlation with PC, showing the oxidation of total protein in plasma, the individual changes in HSA post-race were displayed along with the individual changes in PC (Figs. 3 and 4). For this individual analysis, the criteria used for determining whether there was an increase (or decrease) in HSA oxidation post-race compared to pre-race were: i) HSA oxidation should be increased (or decreased) at all time points post-race compared to pre-race; and ii) HSA oxidation should be increased (or decreased) >20% at least at one time point post-race compared to pre-race. This individual analysis indicated that in some athletes (nos. 6, 8 and 11), HSA oxidation was increased post-race compared to pre-race (Figs. 3 and 4). However, in other athletes (nos. 1, 3 and 5), HSA oxidation post-race was lower than that at pre-race (Fig. 3). In 6 athletes (nos. 2, 4, 7, 9, 10 and 12), HSA oxidation exhibited no changes between pre- and post-exercise (Figs. 3 and 4). The individual comparison between HSA oxidation and PC indicated that in 6 athletes (individuals no. 2, 3, 7, 8, 9 and 10) the changes of the two markers at time points post-race compared to pre-race followed the same trend (i.e., increase or decrease) (Figs. 3 and 4). The two markers are considered to follow the same trend if the difference in the percentage change between them at each time point post-race was <20%. However in 2 athletes (individuals no. 1 and 11), HSA was oxidized more than the total protein, while in others (individuals no. 4, 5, 6 and 12) HSA was more protected from ROS compared with the total protein (Figs. 3 and 4).

Discussion

In the present study, the changes in HSA oxidation were examined following strenuous exercise, such as a mountain-marathon race. Specifically, blood samples were collected from 12 experienced male mountain-marathon runners who participated in a 103 km mountain marathon race, the 'Olympus Mythical Trail'. Blood samples were collected from athletes at four different time points, pre-race and at 24, 48 and 72 h post-race, in order to assess the alterations in their redox status by quantifying HSA dimer formation, that is, HSA oxidation.

HSA is the most abundant protein in plasma, as it makes up approximately 55% of the total serum protein content (34). The function of HSA is based on non-specific binding sites, which allow it to transfer a variety of molecules throughout the circulatory system. Specifically, it binds water, cations (e.g., Ca^{2+}, Na^+ and K^+), fatty acids, hormones, pharmaceuticals and vitamins. The main function of albumin is the regulation of the colloidal osmotic pressure of blood (35). Thus, many of the enzymatic activities of HSA are connected with the binding of metabolic products, which affects the related metabolic pathways (36).

HSA contains a total of 35 cysteine residues from which 34 are involved in intramolecular disulfide bonds and only cysteine34 (Cys34) remains free (37). It is estimated that approximately 70% of the total free thiol content in plasma exists in HSA Cys34 (38). This pool of thiol compounds in plasma gives rise to thiol exchange reactions, leading to a number of disulfide bonds, and thus to the formation of dimers acting as antioxidants by scavenging hydroxyl or other radicals through the reduced sulfhydryl group (39). The dimerization site proved to be the Cys34 by forming a disulfide bridge between two albumin molecules (40). According to Ogasawara *et al* (29), the formed dimers as a result to ROS exposure can be used as an oxidative stress marker. As shown by us and others, the formation of HSA dimers was also displayed following exhaustive exercise-induced oxidative stress (20,41). It is noteworthy

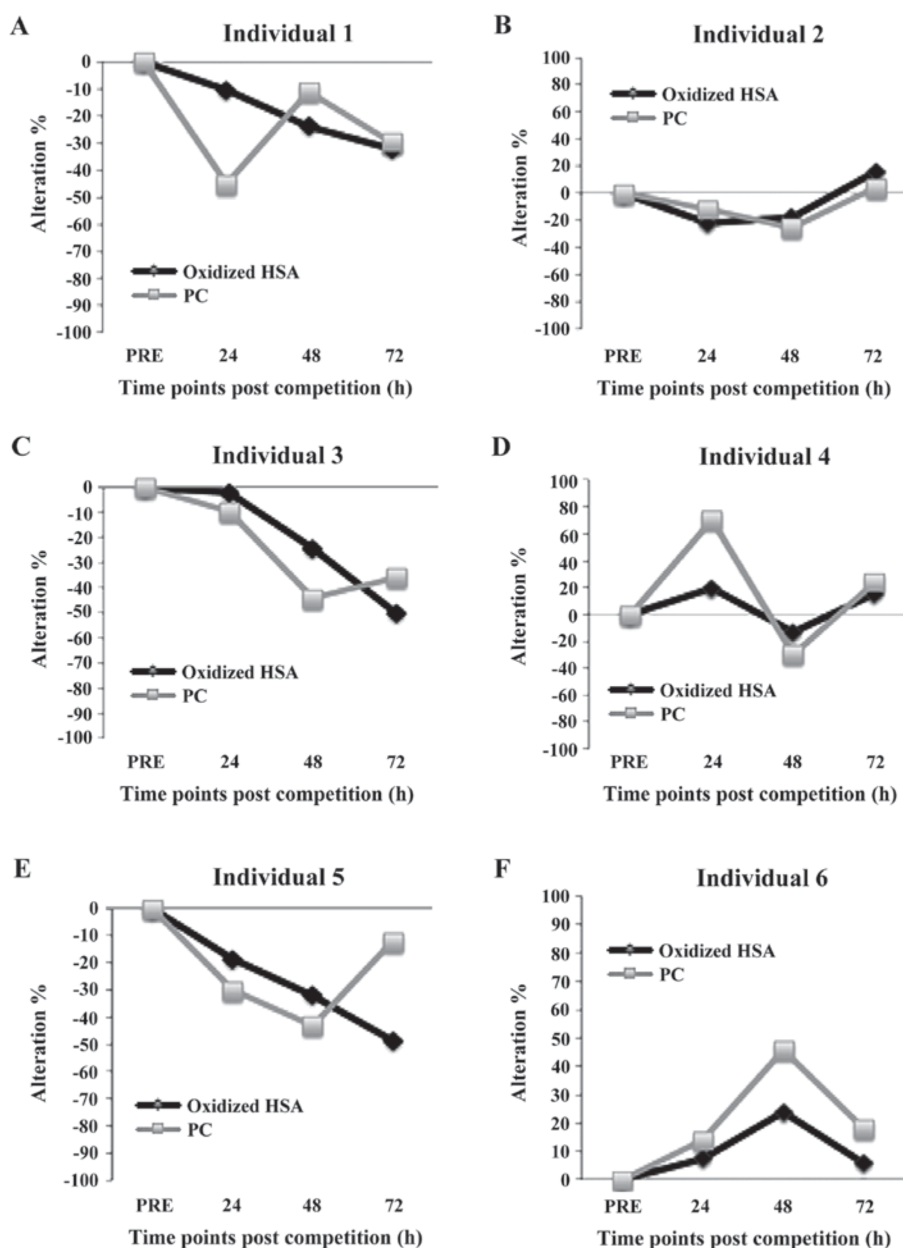

Figure 3. Percentage changes in HSA oxidation (i.e., dimers/monomers ratio), and PC levels of individuals 1-6 at 24, 48 and 72 h post-race time points, compared with pre-race (PRE).

that the exercise-induced increase in ROS activates adaptive responses through signaling pathways regulated by thiol status, including reduced Cys34 of HSA (28,42-44). Additionally, changes in the thiol redox status induce the expression of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), leading to an increase in the levels of the cytokines, IL-6 and TNF-α (42,45). Both of these cytokines not only affect muscle regeneration, but also the development of tolerance following ROS-induced muscle damage. In general, the oxidation of a protein and more specifically HSA following ROS exposure, can lead to a loss of its structural and catalytic function (46). Oxidation and thus dimerization of HSA impedes its activity, as the dimer is more rigid, rendering substrate binding less favorable (47). Specifically, the oxidation of HSA has been observed to decrease both the ligand binding property of site II and the esterase-like activity of HSA, most probably due to conformational changes in subdomain IIIA (47). In addition, HSA has been shown to exhibit an intristic enolase activity towards dihydrotestosterone that is reduced upon dimerization (36). It is known that when oxidized proteins are accumulated in the cells, degradation systems are then activated (48). Moreover, Kawakami *et al* reported that the oxidation of the major antioxidant thiol group Cys34 of HSA results in reduced scavenging activity against highly ROS produced after exercise such as hydroxyl radicals, which may affect athletes' recovery (49). Therefore, it is obvious that excessive HSA oxidation should be prevented. The significant interplay between HSA and oxidative stress necessitates the investigation of HSA oxidation, particularly in athletes undergoing demanding exercise.

The present results indicated that on average, HSA oxidation was not increased at any time point post-race compared

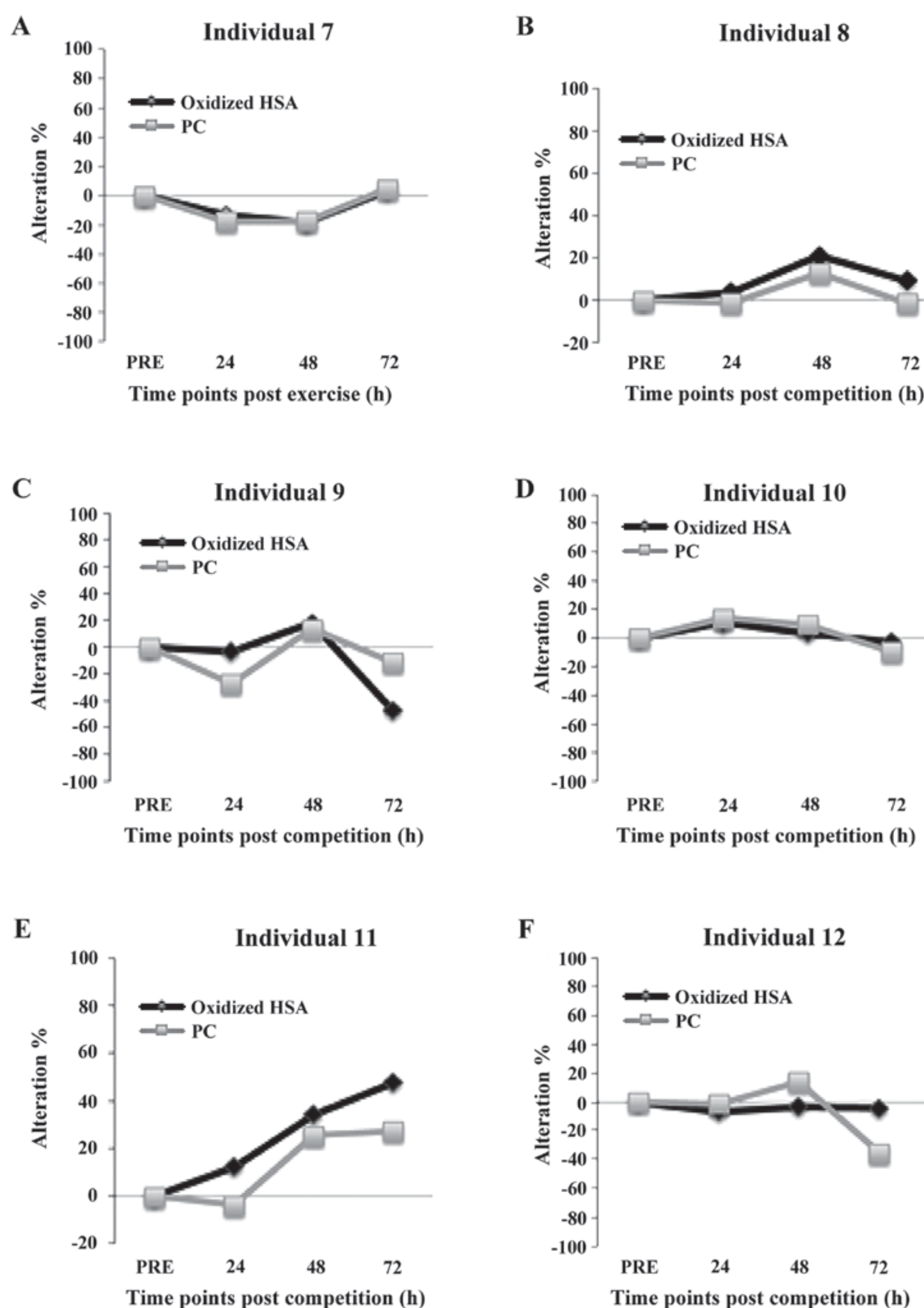

Figure 4. Percentage changes in HSA oxidation (i.e., dimers/monomers ratio), and PC levels of individuals 7-12 at 24, 48 and 72 h post-race time points, compared with pre-race (PRE).

to that at pre-race. Although there was no oxidation of thiols of HSA post-race compared to pre-race, thiol groups of glutathione of the same samples were oxidized as previously demonstrated (31). Therefore, HSA seems to be more protected against oxidation than GSH, since the latter may be involved more in ROS scavenging. Our results were in agreement with those of our previous study on the same samples, which have shown that there was no difference in the oxidation of total protein in plasma between pre and post-race (31). However, other studies have shown an increase in HSA oxidation following exercise (20,28,41). As we have reported previously, this discrepancy between different studies regarding protein oxidation may be explained by the inter-individual variation of protein oxidation after strenuous exercise (31). Likewise, HSA oxidation exhibited great variability, since it was increased in 3 athletes, decreased in 3 athletes and had no change in 6 athletes post-race compared to pre-race.

The samples used in the present study were also analyzed in one of our previous studies by measuring other oxidative stress markers, such as PC, TBARS, TAC, GSH, CAT, sORP and cORP (31). Thus, HSA oxidation was assessed by quantifying dimers, so as to compare its levels with the other performed assays and make conclusions about its usefulness as a biomarker for strenuous exercise-induced oxidative stress. The results revealed a significantly high correlation between HSA oxidation and PC (i.e., oxidation of total protein) at all time points post-race. The aforementioned correlation was expected, since the increased concentration of PC following

exercise has been suggested to be mainly derived from the oxidation of HSA making up approximately 55% of total serum protein, as well as of other major proteins (50,51). In addition, there was a significant positive correlation between HSA oxidation and sORP at 24 h post-race, and a negative correlation with cORP at 48 and 72 h post-race, respectively. These two novel markers have been used previously in several of our studies for assessing exercise-induced oxidative stress (8-11). The above correlations were meaningful, since sORP increased values correspond to higher oxidative stress levels, as it represents the integrated balance of oxidants and reductants, while cORP is a measure of antioxidant reserve available in the body's system.

As mentioned above, HSA oxidation exhibited a great variation between different athletes. Moreover, HSA oxidation had a high correlation with PC, that is, with the oxidation of total plasma protein. Thus, each athlete was examined separately in order to compare the changes in HSA oxidation and PC levels, since HSA constitutes approximately 55% of the total protein content in plasma as mentioned before. Several studies have demonstrated that albumin, as well as fibrinogen are the main protein targets of oxidative stress in plasma (50-53). Moreover, since HSA represents about the half of the total protein content in plasma, a similar trend of PC and HSA oxidation levels in each individual was expected post-race. However, our results indicated that protein oxidation post-race in several athletes exhibited great differences compared to PC levels. Specifically, the results revealed that similar oxidation levels between PC and HSA at all time points post-race were displayed in only 6 out of 12 runners. Thus, in these individuals, it seems that HSA oxidation post-race was not affected differently than the other plasma proteins. However, the remaining 6 athletes exhibited great differences in the changes between PC and HSA oxidation at one or more time points post-race. It was remarkable, that in 4 athletes, HSA was more protected from oxidation than the other plasma proteins. These results supported the hypothesis of Madian and Regnier (54), who suggested that despite the abundance of HSA in plasma, it is not so vulnerable to oxidation as other proteins, such as fibrinogen (53). However, in 2 athletes, HSA oxidation was higher compared to total plasma proteins. To sum up, all these findings suggested that the measurement of only PC is not sufficient to make conclusions about HSA oxidation, and thus it is needed to be examined separately after exercise.

Moreover, in a previous study, we demonstrated that in some of these athletes, instead of protein oxidation post-race, there was protein reduction (31). Similarly, albumin in some of these athletes (individuals no. 1, 3 and 5) exhibited a decrease in oxidation and not an increase post-race. The manifestation of reductive stress instead of oxidative one, particularly after eccentric exercise, has been reported by us, as well as by others (8,55). This intriguing effect can be explained by the high complexity of the regulation of redox homeostasis in human, since many genetic, physiological, biochemical or dietary factors may affect the final outcome of oxidant stimuli (56-58).

The changes in the ratio of dimer to total HSA post-exercise compared to pre-exercise showed the same trend with the ratio of dimer to monomer HSA, that is, in each athlete both ratios either decreased or increased post exercise. Similar to the ratio of dimer to monomer HSA, the ratio of dimer to total HSA exhibited great variability, from 15.42 to 45.37%, between different individuals at all time points post exercise. This variability in HSA oxidation may indicate differences in its functionality, that is, individuals with higher HSA oxidation levels are likely to have lower HSA activity and vice versa. As mentioned above, lower HSA functionality may affect its binding capacity for several ligands, and thus there may be need for a dietary intervention in order to improve the athletes' redox status particularly following strenuous exercise.

In conclusion, the present results support the notion that the assessment of HSA dimers, that is, HSA oxidation may be used as a complementary marker of oxidative stress after exhaustive exercise, particularly as regards the effects on proteins. This inference is supported by the correlation between HSA oxidation and other oxidative stress markers, such as PC, sORP and cORP. In general, thiol levels have been suggested as a marker of oxidative stress (35). However, the assessment of oxidative stress using low molecular weight thiols is difficult, as they are susceptible to oxidative damage and their measurement, particularly in blood, is not easy. Thus, the measurement of stable oxidized thiol groups, such as albumin dimers, is more practical, taking into account that 70% of the total free thiol content in plasma exists in HSA (29). Moreover, the fact that in some athletes the changes in HSA oxidation post-exercise did not follow the changes of PC, suggested the need for assessing both of these markers in order to reach a more confident conclusion about protein oxidation in plasma and make the appropriate dietary interventions. Finally, to the best of our knowledge, this study demonstrated for the first time that in some athletes, HSA was reduced instead of being oxidized post-exercise, highlighting the need for investigating further the individual impact on HSA oxidation and generally in redox status. The understanding of the inter-individual variability of HSA oxidation may prove to be useful for applying the appropriate interventions through nutrition and supplementation to athletes participating in demanding exercise such as mountain marathon race (7,12).

The need for a separate analysis of HSA oxidation is supported by its abundance in plasma and its important physiological roles, which are disturbed after oxidation. This individual approach to HSA oxidation may help athletes to better improve immediate recovery process and consequently health status and performance.

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